Development of influenza vaccines in the face of pandemic threat

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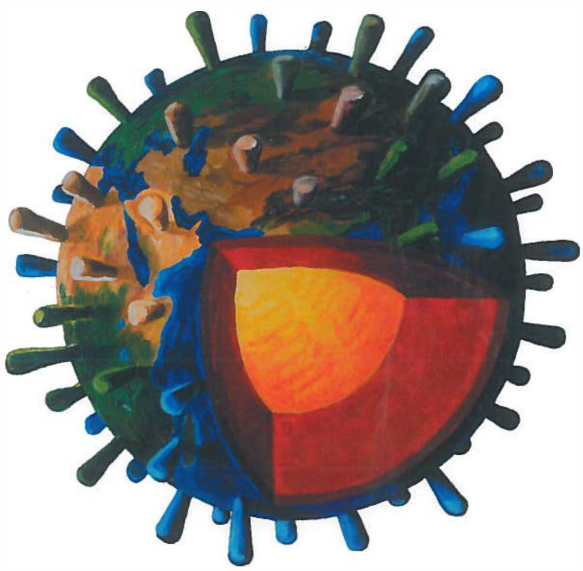
Development *of* Influenza Vaccines *in the Face of* Pandemic Threat



Felix Geeraedts

Development of influenza vaccines in the face of pandemic threat

Stellingen

behorende bij het proefschrift

"Development of Influenza Vaccines in the Face of Pandemic Threat"

Felix Geeraedts, 25 april 2012

1. Gebruik van een geïnactiveerd heel-virus vaccinformulering ten tijde van een influenzapandemie is een efficiënte strategie om de beschikbaarheid van vaccins te vergroten (dit proefschrift).

2. Bij de beoordeling van influenzavaccins moet, naast de hoeveelheid opgewekte antistoffen, ook de kwaliteit van de immuunrespons in de afweging betrokken worden (dit proefschrift).

3. Aangezien adjuvantia onvoorziene bijwerkingen kunnen hebben, die juist bij vaccinatie van grote massa's zichtbaar worden, en sommige adjuvantia zelfs de beschermende werking van een vaccin kunnen verminderen, is het raadzaam het gebruik van (deze) adjuvantia voor pandemische griep te minimaliseren.

4. Activatie van het aangeboren immuunsysteem via specifieke receptoren speelt een belangrijke rol bij vaccin-geïnduceerde adaptieve afweer (dit proefschrift).

5. De partikelstructuur van het influenza virus, inclusief het virale RNA, dient behouden te blijven voor een optimale immuunrespons (dit proefschrift).

6. Vriesdrogen van geïnactiveerd heel-virus influenzavaccin met stabiliserende suikers vergroot de mogelijkheden voor opslag en snelle verspreiding van vaccins om een beginnende pandemie in de kiem te smoren (dit proefschrift).

7. De relatie tussen pathogene micro-organismen en ons immuunsysteem zou je kunnen samenvatten met de bekende uitdrukking 'als je er niet dood aan gaat wordt je er alleen maar sterker van'.

8. Wat betreft de vraag of het mogelijk is om in de tijd te reizen blijkt de kink in de kabel toch niet alleen in ons voorstellingsvermogen te zitten.
(Error Undoes Faster-Than-Light Neutrino Results, http://news.sciencemag.org /scienceinsider/2012/02/breaking-news-error-undoes-faster.html)

9. Concurrentie in de gezondheidszorg door de invoering van winstuitkering kan tot innovatie leiden, maar maakt ook dat nieuwe inzichten niet meer snel gedeeld zullen worden, waardoor dit beleid in zekere zin een dooddoener is.

10. Van de dosis-sparende influenza vaccins is geïnactiveerd heel-virus vaccin het beste adjuvans-sparende vaccin.

11. Je hebt een microscoop nodig om de relatie tussen arts-microbioloog en patiënt goed zichtbaar te maken.

12. '*Nature is very predictable when properly understood*' (Big wave surfer Laird Hamilton, in SURF magazine 5, juli 2007).

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Development of influenza vaccines in the face of pandemic threat

Proefschrift

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aan de Rijksuniversiteit Groningen
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Felix Cosmas Gerard Geeraedts

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te Nijmegen

Promotores:	Prof. dr. A.L.W. Huckriede
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Copromotor:	Dr. A. de Haan
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	Prof. dr. G.F. Rimmelzwaan

The research described in this thesis was performed at the Department of Medical Microbiology, Molecular Virology Section, of the University Medical Center Groningen within the Groningen University Institute for Drug Exploration (Guide). Part of the work was performed in collaboration with the Department of Pharmaceutical Technology and Biopharmacy of the University of Groningen, and the Department of Medicine, Division of Infectious Diseases and Immunology of the University of Massachusetts Medical School, Worcester, Massachusetts, United States of America.

The conducted studies were part of the activities of the Netherlands Influenza Vaccine Research Centre (NIVAREC). The objectives of NIVAREC were stated as follows:

The primary objective of the NIVAREC program is to establish a virtual research centre in which academic groups active in the area of influenza virus biology and vaccine development join forces with a leading influenza vaccine manufacturer in synergistic fashion in order to contribute to an optimal preparedness in The Netherlands for a situation in which a new influenza pandemic would strike.

The NIVAREC partners are the Department of Medical Microbiology, Molecular Virology Section, University Medical Center, Groningen (UMCG), the Department of Virology, Erasmus MC, Rotterdam (EMCR) and Solvay Biologicals, Weesp (recently acquired by Abbott).

The NIVAREC consortium will specifically focus on the following key areas:

(i) generation of novel recombinant influenza virus strains covering a broad spectrum of currently known influenza viruses with pandemic potential,

(ii) developing technology for the rapid production of vaccine viruses on an industrial scale

(iii) developing innovative technology for the generation of safe and efficacious influenza virus vaccines that can be manufactured rapidly and on a large scale when required.

Emphasis in the research activities of NIVAREC will be on the H5N1 ("bird flu") virus, since this virus at the start of the NIVAREC program posed, and continues to pose, a serious influenza pandemic threat.

The work described in this PhD thesis was performed under the auspices of the NIVAREC, with financial support from the Netherlands Organization for Health Research and Development (ZonMw). Funding for a part of the study was obtained from the Jan Kornelis de Cock Foundation.

Contents

GENERAL INTRODUCTION AND AIM OF THE THESIS

Influenza pandemics

An influenza pandemic is a sudden global outbreak of the disease which occurs at an unpredictable time and causes excess morbidity and deaths [1]. In the past 300 years, about 11 influenza pandemics have occurred at intervals ranging from approximately 10 to 50 years [2,3]. The pandemics of modern history are the 1918 'Spanish flu', 1957 'Asian flu', 1968 'Hong Kong flu', and 2009 'Mexican flu'. The causative agent is an influenza A virus strain, other than the seasonal strains circulating among humans just before the outbreak [4]. Influenza virus is a highly transmissible pathogen which primarily infects the epithelial cells of the respiratory tract. The clinical presentation may vary from asymptomatic to flu-like symptoms (fever, myalgia, dry cough, headache, chills, diarrhea), to life-threatening viral pneumonitis, and secondary bacterial pneumonia [5]. Influenza epidemics are responsible for an estimated 250,000 to 500,000 deaths worldwide each year [6a]. There may be a large variation between flu seasons with death rates ranging from 3,000 to 49,000 in the US alone [6b]. Excess deaths due to pandemics also varies considerably from 18,000 in 2009, which is likely an underestimation, up to 50-100 million in 1918 [7-9]. Where most casualties of epidemic influenza concern the very young and the elderly, pandemic influenza may show high lethality in young healthy adults, as was the case in 1918. The rapid death of these young adults has been explained in part by a 'cytokine storm' resulting from a severe immune response to an unusually virulent virus [10].

Biology of influenza virus

Influenza virus is an enveloped single stranded (ss)RNA virus which belongs to the family of *Orthomyxoviridae* [11,12]. It is further classified in type A,B and C, of which only influenza A is known to cause pandemics. The viral genome of influenza A consists of 8 RNA segments containing 11 genes, encoding for 11 proteins: hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), RNA polymerase acidic protein (PA), and basic protein 1 (PB1), 1-F2 (PB1-F2) and 2 (PB2), non-structural protein 1 (NS1), and 2 or nuclear export protein (NS2 or NEP), and matrix protein 1 (M1), and 2 (M2). The RNA segments together with NP and the polymerase subunits form the ribonucleoproteins, which together with the M1 protein constitute the viral core [11, 13]. The core is surrounded by a lipid

envelop membrane, derived from the host cell, to which it is linked via M1 [11]. The HA and NA spike proteins are located in the membrane and project from the lipid envelop into the external environment. Small amounts of M2 are present in the membrane. NS2 is also associated with the viral particle (Figure 1). NS1 is not represented in the viral particle but produced in the host cell upon infection.

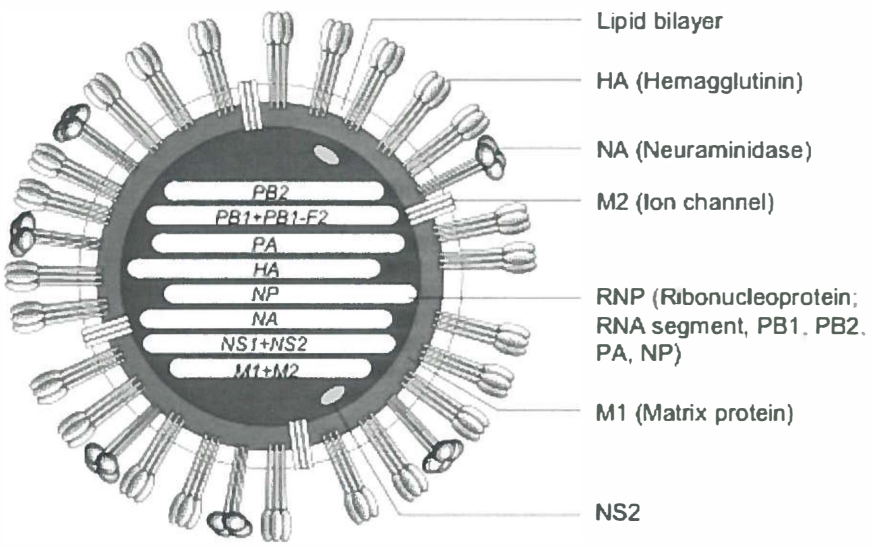

Figure 1. *Schematic model of influenza A virus.*

Influenza virus enters the host cell by binding of the HA to sialic-acid receptors on the cell surface, followed by endocytosis [14]. The virus is further trafficked to late endosomes, where the acidic pH of the endosomal lumen initiates a conformational change in the HA molecules, which triggers fusion of the viral membrane with the endosomal membrane[14]. Additionally, the low pH initiates the process of uncoating, by proton influx through the M2 ion channels in the viral membrane, resulting in dissociation of the RNP from M1 and release of the viral RNA into the cytoplasm, after viral membrane fusion has occurred [13]. The RNA is transported to the nucleus, where it is transcribed and replicated. Synthesis of viral proteins occurs in the cytosol. New viral particles are assembled at the plasma membrane and subsequently released from the cell by budding. The viral NA plays an important role in the release of off-spring virus from the cell surface.

The function of NS1 is to counteract antiviral type I interferon production by the host cell.

HA and NA are important antigens and antibody responses to HA confer protection against infection [11]. Currently, 16 antigenically distinct HA and 9 NA subtypes are known, and an influenza virus subtype is denoted by a number combination referring to its HA and NA subtype. The virus subtypes that caused the pandemics of 1918, 1957, 1968, and 2009 were H1N1, H2N2, H3N2, and H1N1 respectively [15,16]. Birds are the main reservoir for all influenza A virus subtypes [17, 18], and numerous different combinations of the 16 HA and 9 NA subtypes can be found in wild fowl and poultry [19]. Other natural hosts to influenza virus include pigs, and horses [17].

Influenza A virus exhibits a high frequency of antigenic variation. Firstly, new influenza viruses with a different HA may be introduced in the human population, which causes an 'antigenic shift'. Lack of pre-existing immunity to this new HA promotes the rapid spread of the virus, which results in a pandemic. The pandemic virus replaces the previously circulating epidemic virus. Secondly, point mutations in the viral RNA easily occur during replication, due to the absence of a proofreading system. In circulating influenza strains, these point mutations accumulate and gradually alter the antigenicity so that a previously immune person becomes susceptible again, every 3-5 years [29]. This process is called 'antigenic drift'.

Origin of pandemic viruses

Pandemic viruses in general originate from an animal source, notably birds or swine. This is confirmed by molecular analysis of strains from pandemics in the era of modern virology [20-22]. Isolation of influenza virus first became possible in 1933 [23]. The genomic RNA sequences of the notorious 1918 virus, which seemed to be lost to time, have recently been recovered from archived materials, and from frozen samples of victims buried in the permafrost [24,25]. The 1918 RNA sequences point to an avian and/or swine origin of the Spanish flu virus [26,27].

Animal viruses may be introduced in the human population through gene-reassortment with human influenza viruses, or directly from an animal progenitor virus, as has also been argued for the 1918 virus [26, reviewed in 28]. Gene-reassortment is the process of gene exchange between two different viruses, for instance an avian virus and human virus, which may occur when they

simultaneously infect the same host cell [11]. This process is facilitated by the segmented nature of the RNA genome, and can lead to the generation of a new virus. The 1957 and 1968 viruses were reassortants between circulating human viruses and avian viruses. The 1957 virus obtained its H2, N2, and PB1, and the 1968 virus its H3, and PB1 from avian viruses [28]. The 2009 virus was a triple re-assortant virus, containing genes of swine, avian and human influenza viruses that were already circulating in swine [28, 22]. Obviously, the 2009 virus entered the human population without the direct involvement of a human influenza virus [28].

World-wide outbreaks caused by human influenza viruses have been either disqualified by definition or disputed for other reasons as being a true pandemic. These events include the outbreak in 1947/48 of what turned out to be a major drift variant of the circulating human H1N1 virus, while the pandemic definition excluded drift variants as causative agent, and the reintroduction in 1977 of an H1N1 strain which was identical to the strain which had been circulating among humans in the 1950s [4,5]. Where this virus came from is unknown, perhaps some institutional freezer [4]. The H1N1 also did not replace the pre-existent epidemic virus. It started to co-circulate with the H3N2 that was already circulating since 1968 [4,15]. Although the outbreak fulfilled the criteria of the pandemic definition at that time, which included the requirement of a new subtype introduction [4], it was still disputed if it was a true pandemic (Textbox 1).

UNPREDICTABILITY OF PANDEMICS

It is not known when a next pandemic will occur, or what virus subtype will be the cause of it. In the past decade human H2N2 as well as a number of different avian influenza subtypes, including H5, H6, H7 and H9 were thought to be likely candidates [10]. The 2009 swine H1N1 pandemic therefore came as a surprise [16]. With an H1N1 already circulating in humans, the introduction of a similar subtype seemed rather unlikely, but obviously, the human H1N1 had not generated sufficient cross-protective immunity to avoid a pandemic [30]. Still, pandemic preparedness in 2009 was better than ever before, due to the pandemic threat created by the cross-species transmission of a particular avian influenza virus, which had generated a surge in pandemic vaccine technology and knowledge in the preceding years [31, 32].

Textbox 1: Until 2009, indisputable pandemics have been caused by newly introduced viruses from an animal source [15]. In 2009 a swine-origin H1N1 virus was introduced, followed by a rapid worldwide spread, while at that moment an H1N1 was already circulating in the human population since 1977. One might question the requirement for a new subtype virus in the pandemic definition, as it would disqualify this event as a true pandemic. Recently, a new broader definition has been proposed which circumvents this issue [4]. One of the criteria is that 'the new and spreading influenza A virus has an HA which does not cross-react in HI tests with the HA of the immediately preceding and circulating influenza virus strain(s)'. This also allows for drift variants like the 1947 virus to be included, but might diminish the distinctive character of pandemic influenza as opposed to its epidemic form. Perhaps, a pandemic should be defined just by the behaviour of the virus in the population and the term true pandemic virus be reserved to pandemic viruses with an animal origin. Obviously there are more pandemics than true pandemic viruses.

The H5N1 pandemic threat

The first time that highly pathogenic avian influenza (HPAI) H5N1 virus was encountered in humans was in 1997 during an outbreak in Hong-Kong. Eighteen persons became infected of whom six died [33,34]. The human cases occurred amidst large outbreaks of HPAI H5N1 among poultry on live animal markets and farms in Hong-Kong, where it was 70 to 100 percent lethal in chicken [33]. It soon became evident that the human cases had contracted the virus from birds [33]. The second occasion in which H5N1 appeared in humans occurred early in 2003, when three persons from Hong-Kong, who recently travelled to the Fujian province of China, became ill with H5N1. Two of them died [35]. So far, these two events concerned isolated incidents. However, at the end of 2003 through early 2004 Korea, Thailand and Vietnam started to report of continuous outbreaks of HPAI H5N1 among poultry, and reports of human H5N1 cases with high fatality rates followed in January 2004 in Vietnam and Thailand [35]. These outbreaks could not be contained, and since then H5N1 has spread to at least 15 countries causing an ever increasing number of laboratory confirmed H5N1 human infections, 562 up

to this time of writing, with a case-fatality rate approaching 60% [39] (Textbox 2, 3 and 4).

Textbox 2. In 2006, a fatal case from Bejing back in november 2003, which had first been diagnosed as severe acute respiratory syndrome (SARS), was re-examined. SARS is a potentially lethal complication of a coronavirus (SARS-CoV) infection. The true causative agent, however, turned out to be HPAI H5N1 virus. In retrospect, this casualty marks the beginning of the currently ongoing episode of (sporadic) bird to human transmissions with HPAI H5N1 [1,3].

Textbox 3. In 1997, containment measures included the culling of all poultry (1.5 million chicken) in Hong-Kong which effectively eliminated the virus from sight [36], although occasionally new H5N1 genotypes kept reappearing in poultry in Hong-Kong, despite of stringent control-measures [34,36,37]. Similar efforts to eliminate the virus by massive culling of poultry in Vietnam and Thailand in March 2004 led to a decline in cases [38]. But this time this drastic measure proved insufficient to contain the situation, and soon hereafter the number of H5N1 cases started to increase again and the virus started to spread to other countries in Asia [38,39].

Textbox 4. Spread of HPAI H5N1 likely occurs through trade of infected poultry and transport of infected materials. Other factors that played essential roles in the occurrence and dissemination of H5N1 are domestic waterfowl, specific farming practices, and agro-ecological environments [19]. HPAI H5N1 has been found in many wild migratory birds, also in several European countries. Its lethality in these birds is variable and it is argued that H5N1 might be spreading by migration. Yet, whether these infected birds represent an important vector remains to be proven [19].

HUMAN-TO-HUMAN TRANSMISSION

As the H5N1 outbreak turned beyond control, the chance that human-to-human transmission would evolve also increased. It was feared that the virus might adapt to its new host or reassort with a human influenza virus to become highly transmissible. Indeed, there is evidence that limited human-to-human transmission may have occurred in household clusters [40,41]. Yet, none of these transmissions became sustained. Human-to-human transmission may be inefficient because avian influenza viruses, unlike human virus, generally do not bind to alpha-2,6Gal sialic acid receptors located in the human upper respiratory tract, but instead preferentially attach to the alpha-2,3Gal receptor type, which is restricted to the lower respiratory tract in humans [42,43] (Textbox 5.).

Textbox 5. Which mutation would turn H5N1 into a human influenza virus remains unknown, but a mutation that changes its receptor specificity is highly suspected [44]. The avian derived H2 and H3 of the pandemic viruses of 1957 and 1968 have mutations that changed their receptor specificity to the human-type alpha-2,6Gal receptor. Similar mutations experimentally applied to H5N1 have been shown to shift the virus preference to the alpha-2,6Gal receptor [44]. If such a mutation would occur, H5N1 might find foot-hold in humans [44]. It has however been argued by others that mutations in the HA gene on itself may not be enough to allow for efficient spreading among humans, and that an additional mutation in the PB2 gene of H5N1, which increases viral replication in human cells, may be needed [45a]. It has been recently announced on the fourth ESWI Influenza Conference in Malta that an airborne H5N1 virus has been deliberately created in a laboratory by infecting ferrets with a mammalian adapted version of H5N1 bearing 3 mutations, and then passing this virus 10 times from ferret to ferret. Only a total of 5 mutations in two genes were required to induce transmissibility by aerosols in ferrets. Even more troublesome, the virulence of the virus remained undiminished [45b].

VIRULENCE FACTORS

Although a case-fatality rate of 60% H5N1 may be an overestimation as subclinical infections seem to occur [46], H5N1 is an extremely virulent virus, with the primary cause of death due to H5N1 infection being progressive respiratory failure [47,48]. The mechanism responsible for the virulence of H5N1 in humans is largely unknown. Restriction of the alpha-2,3Gal receptor, to which H5N1 preferentially binds, to the *lower* respiratory tract in humans might play a role in severe pulmonary disease once infection is established [48]. High viral replication and concurrent induction of an imbalance in the cytokine response with increased production of inflammatory cytokines probably play key roles in the virulence of H5N1 in humans [50,53,54] (Textbox 6.).

Textbox 6 In birds, HPAI H5N1 pathogenicity is characterized by a broad tissue tropism and systemic replication, which correlates with the presence of a multibasic cleavage site in the HA [reviewed in 49]. In humans H5N1 has been detected outside the respiratory tract [50,51] yet, the role of the multibasic cleavage site in H5N1 pathogenicity in humans remains unclear. Insertion of a multibasic cleavage site in human H3N2 virus on itself seems not enough to confer increased pathogenicity in a ferret model [52]. More likely, multiple viral proteins may be involved in H5N1 pathogenicity in mammals, including HA, PB2 and NS1, and different gene constellations can confer high pathogenicity [49].

STATE OF PANDEMIC ALERT

The H5N1 virus capers forced the WHO to raise the pandemic alert to level 3, which is one step away from a beginning pandemic [55]. This situation has remained so from 2004 up to today. The pandemic alert level was only altered by the arrival of the 2009 pandemic and raised to level 6, to fall back to level 3 again afterwards. Yet, the only obstacle separating the world from a beginning H5N1 pandemic on the WHO scale is sustained human-to-human transmission, and the high fatality rate of H5N1 infection makes one fear for the worst if this virus

would set off a pandemic [56]. This ongoing H5N1 pandemic threat urges the development of effective pandemic mitigation strategies.

Antivirals and vaccines

Use of vaccines and antiviral drugs plays a key role in pandemic mitigation [57,58]. This type of intervention is considered clearly efficacious in preventing infection and treating illness, where the role for other measures like closing schools, wearing face masks, and isolating infected people from the uninfected still needs further investigation [59] (Textbox 7.).

ANTIVIRAL DRUGS

Two types of antiviral drugs are registered for use against influenza, M2 proton channel inhibitors (amantadine and rimantadine) and NA inhibitors (oseltamivir and zanamivir).
Although highly resistant to M2 inhibitors [49], HPAI H5N1 was found susceptible to NA-inhibitors in vitro [60,61]. The therapeutic merits of oseltamivir were, however, doubtful in the first H5N1 patients [40], but early treatment may be beneficial for the clinical outcome and improve survival [62]. Oseltamivir may be used as a prophylaxis, as it was proven to prevent the initiation of influenza virus infection of airway epithelial cells in vitro [63]. However, prophylactic use of antiviral drugs provides only short-term protection and there is a risk of emergence (and selection) of oseltamivir resistant strains by improper drug use [64-66]. Especially in the early phase of a pandemic, antiviral drugs may be the primary pharmaceutical resource to rely on, in expectation of suitable vaccines [49]. However, if containment fails, prophylactic application of antivirals will become a very inefficient way to use the limited resources [67].

INFLUENZA VACCINES

In contrast to antivirals influenza vaccines provide long term protection against infection, and are the mainstay of prevention and control of epidemic and pandemic influenza [68,32].

Current vaccine formulations

Currently licensed influenza vaccines can be roughly divided in inactivated vaccines and live attenuated vaccines. Inactivated vaccine formulations include whole inactivated virus (WIV) vaccine, split-virus vaccine, subunit-vaccine and virosomal vaccine. Live attenuated H5N1 vaccines have been developed, but are not likely being used until the virus is wide-spread, in order to keep the introduction of new influenza genes in the human population at the lowest level. Consequently, development of H5N1 vaccines has been largely focused on the inactivated vaccine formulations [69]. Other types of influenza vaccine in development include virus-like particles, DNA-vaccine, and recombinant-HA vaccine [70].

Inactivated vaccine production

Production of inactivated influenza vaccines basically starts with bulk production of influenza virus, which is grown in embryonated chicken eggs or cell culture. To this end a seed-virus is used that carries a combination of genes which encode for the specific antigens, and genes which promote high-yield growth. Conventionally, a seed-virus is a reassortant obtained by simultaneously infecting chicken eggs with the specific virus and an egg-adapted strain [70].

After bulk production of vaccine virus, the virus particles are purified and inactivated, using formaldehyde or beta-propiolactone. These particles can be used directly as a WIV vaccine. Alternatively, the virus particles may be degraded by treatment with detergent and/or ether to produce a split-virus vaccine. Further purification of the HA and NA proteins yields a subunit vaccine [71]. A virosomal vaccine is produced by detergent treatment, followed by removal of the nucleocapsid containing the viral RNA, and reconstitution of the viral envelop membrane bearing the HA and NA proteins [72].

Vaccine immunogenicity

The protective efficacy of a vaccine is primarily determined by the induction of neutralising antibodies against the viral HA surface protein. Antibodies against NA play a secondary role but may contribute to protection and may lessen the severity of infection [73]. Consequently these antigens are the essential components of all inactivated influenza vaccines. A sufficient amount of vaccine-induced serum anti-HA antibodies, indicated by a serum hemagglutination inhibition (HI) titre >40, is generally accepted as the most important correlate of

protection [74,75]. In addition, cytotoxic T lymphocyte (CTL) induction may play a role in cross-protective immunity and could aid by reducing disease severity and mortality in a pandemic [75].

Textbox 7. Mathematical models have been employed to predict the result of different containment measures including chemo-prophylaxis, household quarantine and pre-vaccination on the outcome of an emerging H5N1 pandemic in South-East Asia. [76,77]. All three measures applied together and well in time could contain a pandemic at the source when the R0 (mean number of secondary cases infected by one infected person) is as high as 2.4. This would impose controllability over potential pandemic viruses, as infectious as the 1918 virus with an estimated R0 of 2 to 3, and is an argument for the stockpiling of oseltamivir and pre-pandemic vaccines for containment actions [78,79].

Pandemic vaccine

The ideal vaccine against an influenza pandemic would be one that is available at the onset of the pandemic, in quantities sufficient to vaccinate the majority of the world population, and sufficiently potent to allow for one immunization for protection. Although influenza vaccines have been in development for a long time, starting in the 1940s, such vaccine does not exist.

Challenges in pandemic vaccine production

Knowledge about the exact virus subtype and strain is crucial to produce a protective vaccine [73], but will only become available once the pandemic has started. Then, it will take 4 to 6 months of production time before the first vaccines become available, while it may take only 2 months for the virus to spread worldwide [79, 80]. Speeding up the production process is therefore paramount (this problem was evident in the 2009 pandemic [81]). H5N1's lethality to humans and chickens poses a safety concern, and hampers the production on chicken eggs. The global production capacity is limited and owned by only a few industrialized countries, which creates a vaccine shortage and may lead to uneven distribution

[56] (and in fact did so during the 2009 pandemic [82]). Besides the search for ways to expand production capacity, high priority should therefore be given to the investigation of strategies that economise on the use of antigen [83]. As a complicating factor, H5N1 virus processed into conventional vaccines is only weakly immunogenic in un-primed humans [71,84]. Furthermore, because there is no pre-existing cross-protective immunity in the population to H5N1, an extra priming dose is required [84]. Vaccines produced and stockpiled in anticipation of a pandemic need to be cross-protective, because the strain that actually causes the pandemic may have drifted away from the vaccine strain [79]. Also, because it is unknown when the next pandemic will occur, the stability of the stockpiled vaccines over time is an important issue.

Rapid production of low-pathogenic, high-yield seed strains

The majority of the vaccines are produced on embryonated chicken-eggs, which are killed by the H5N1 virus. A new technique called reverse-genetics made it possible to delete the nucleic acid sequences that confer the pathogenicity traits (at least for chickens and eggs) from the HA gene, and reconstruct a virus de novo using DNA plasmids, which provide the genetic information for the RNA segments of the viral genome. [85]. By combining the modified H5N1 HA and the NA gene with the other genes of an attenuated strain (PR/8) which has been extensively used in vaccine production, a high-yield non-pathogenic vaccine virus can be created with on its surface the HA and NA proteins of H5N1. This way, recombinant seed viruses for vaccine production can be readily made and this strategy has been used to produce H5N1 pre-pandemic vaccines [86-88].

Using reverse genetics to produce a seed virus will also speed up vaccine production in response to a pandemic. Normally, an 8-12 week period is necessary to produce a safe vaccine strain from the wild type virus, through reassortment in double infected embryonated chicken eggs [89, 70]. With reverse genetics a vaccine virus can be generated in less than 4 weeks [90]. Availability of vaccines will also be accelerated by fast tract licensure based on the experience with mock-up vaccines using a comparable virus that simulates the novelty of a pandemic virus [89].

Limitations in mass vaccine production and low vaccine immunogenicity

In a standard dose of seasonal subunit vaccine 15 μg of HA of a particular subtype is incorporated, providing 70-90% protection in healthy adults [90]. In contrast,

two doses of 90 μg of H5N1 subunit vaccine were needed to reach seroprotection in >50% of the recipients [84, 91]. The low immunogenicity of H5N1 combined with a limited global vaccine production capacity necessitates the development of dose-sparing strategies. Possible dose-sparing strategies include the use of WIV vaccines and addition of adjuvants.

WIV vaccines became outdated for epidemic use, due to the development of lower reactogenic split-virus and subunit vaccines, they are however more immunogenic in un-primed individuals than unadjuvanted split-virus and subunit vaccines [92-94], and the production process is less complicated and could potentially be faster.

Adjuvants in potential augment the immunogenicity of a vaccine. Currently, few adjuvants are licensed for use in humans. Only aluminum hydroxide is US FDA approved. The European Medicines Agency has however approved the newer and stronger adjuvants MF-59 in combination with inactivated influenza vaccines (in 1997), and ASO3 (in 2009) in combination with H1N1 pandemic vaccines [89].

Dealing with absence of pre-existing immunity

In case of the 2009 H1N1 pandemic, vaccination with a single dose of 15 μg HA seemed sufficient to induce protective antibody responses in >93% of healthy young adults [95,96]. Previous exposure to related virus strains may have primed the immune system for a rapid and effective antibody response [73]. In case of H5N1, there is no pre-existing immunity, because this virus is only remotely related to the human influenza strains and is not frequently encountered in humans. Clinical studies with H5N1 vaccines have shown that two immunization are necessary for a protective antibody response [84,86-88]. For pandemic mitigation this is unfavourable as it will cost time and valuable vaccine doses. One way to deal with this problem is to use stockpiled pre-pandemic vaccines and administer the first dose as early in the evolving pandemic as possible.

There is, however, a fair chance that stockpiled H5N1 pre-pandemic as well as pandemic vaccines may not match the actual pandemic virus, due to the evolution of drift variants. Since 1997, 10 antigenically distinct H5N1 clades have evolved (numbered from 0-9) [62] of which multiple clades (0, 1, 2 (subclade 2.1 to 2.3), and 7) have caused disease in humans. Promisingly, pre-pandemic H5N1 vaccines containing whole virus particles or oil-in water adjuvants have been shown to induce significant cross-protective antibody response against viruses

from different H5N1 clades [86,87,98-102]. Also, there is some evidence that a poorly-matching pre-pandemic vaccine could prime for the correct matching vaccine and may be administered in advance of the pandemic wave [97]. Using this strategy protective antibody levels can be reached earlier, and matching vaccines are being spared.

Limited storage stability

Pre-pandemic vaccines seem to have a role in pandemic mitigation due to their cross-protective activity and potential to serve as a priming agent. These features promote the stockpiling of pre-pandemic vaccines. In the past decade H5N1 vaccines have been stockpiled by nations as well as the WHO for containment purposes and resource-poor countries [79]. The shelf live of a vaccine is however limited and vaccines stockpiled some years ago may already have reached the expiry dates. For long-term storage and minimal vaccine wastage vaccine stability is an important issue. Additionally, because of their immediacy and world-wide application pre-pandemic as well as pandemic vaccines preferably need to be temperature stable, with a minimal requirement for cold-chain handling and storage [89].

IMPROVING PANDEMIC VACCINES

To tackle some of the challenges of pandemic vaccine development, new approaches to vaccine immunogenicity and stability may be explored.

Enhancing the quality of the antibody response

The efficacy of a vaccine is traditionally measured by the level of serum HI antibodies induced by the vaccine. However, also the type of immune response induced, in terms of Th1 or Th2 type response, may be important for protective immunity. Influenza virus infection naturally induces a Th1 type response which coincides with production of specific subtype antibodies [103]. This response-type best provides protection against reinfection with influenza virus [104]. In mice Th1 type antibodies have been shown to protect better against infection than Th2 type antibodies [105,106]. In some cases a wrong response type can be detrimental. A biased Th2 type response to a Respiratory Syncytial Virus (RSV) vaccine has been associated with vaccine-enhanced disease after RSV infection [107]. Differentiation into either Th1 or Th2 cells, after the activation of T cells by dendritic cells,

strongly depends on the presence of Th1-inducing cytokines, like interleukin 12 (IL-12) and interferon α (IFNα), or Th2-inducing cytokines, like IL-4 and IL-10, respectively [108].

Exploiting innate immunity

In the past decade there has been a growing interest in mechanisms by which the innate immune system steers the adaptive immune responses [108]. Upon invasion, microbes are first recognized by pattern recognition receptors (PRR) of the innate immune system that sense microbial components, known as pathogen associated molecular patterns (PAMP) [109]. Viral nucleic acids are important PAMPS, with their PRRs being Toll-like receptors (TLR), which are located in the endosomes, and intracytoplasmic receptors like retinoic-acid-inducible protein I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and DNA-dependent activator of IFN-regulatory factors (DAI) [110]. Intracytoplasmic PRRs are ubiquitously expressed and generally recognize viral nucleic acids produced during viral replication. The TLRs are restricted to specific cell subsets of the immune system, like dendritic cells (DC), NK cells, T cells and B cells, and recognize viral double stranded (ds) RNA (TLR3), single stranded (ss) RNA (TLR7 and TLR8), and DNA (TLR9) [110]. PRR activation by invading virus induces a signalling cascade resulting in activation of NF-κB and IRF leading to production of proinflammatory cytokines and type I IFN (IFNα/β), of which the latter is a direct inducer of an anti-viral state in the infected and neighbouring cells. [109]. In the TLR signalling cascade, MyD88 is a crucial adapter molecule for all TLRs except TLR3.

It has become increasingly clear that activation of TLR is an important factor leading to the induction of the specific cytokine profiles that shape the response type into Th1 or Th2 [108]. DC's play a crucial role in linking the innate with the adaptive immune system. TLR7 activation in plasmacytoid dendritic cells (pDC) induces a strong IFNα response, whereas conventional DC (cDC) hardly produce IFNα to TLR7 stimulation, but instead produce IL-6, IL-12 and TNFα [110]. Besides its role as Th1-inducing cytokine, IFN-I is important for antibody class switching of B cells, and stimulates cross-priming [110]. In addition to DC activation TLR7 triggering has a direct effect on antibody production by B-cells [111].

Improving vaccine stability

In general, protein pharmaceutics, like vaccines, in aqueous solution are prone to physical and chemical degradation [112]. Liquid influenza subunit vaccine may be kept outside the fridge for only a limited time, which is in the order of a couple of weeks, before the HA starts to degrade and the shelf-live becomes affected [113]. Dry-powder vaccines are more stable, and can be stored at ambient temperature for considerably longer periods [114,115]. Such increased stability would benefit pandemic use.

Freeze-drying is a method that has been successfully used to produce dry-powder influenza vaccines, including WIV, split-virus, subunit, and virosomal vaccine [116-119]. To avoid detrimental stresses on the HA during freezing and drying and to stabilize the vaccine during storage, disaccharide or oligosaccharide sugars can be added [reviewed in 120]. When the sugar solution containing the vaccine is rapidly frozen (in liquid nitrogen), water crystals will form whereas the crystallization rate of the sugar is too slow to form crystals. This leads to (freeze-) concentration of the sugar (and vaccine compound) and consequently further depression of the freeze-point of water by the solute. When the freeze-concentration reaches its maximum, which is at the glass-transition temperature, viscosity dramatically increases and an amorphous glass is formed in which water, sugar and the vaccine compound are immobilized, and in which water is prevented from crystallization, during further lowering of the temperature. Subsequently all water, including crystals that have been formed, is removed by sublimation in a vacuum. During drying the temperature needs to remain below the transition point to a 'rubbery' state (Tg'), where the stabilization by the glassy matrix becomes compromised, and crystallization of the sugar may occur. The Tg' depends on the water, sugar, and vaccine compound in the glass, and increases with the loss of water. In the end a dry amorphous sugar glass is formed in which the vaccine compound is captured. This can be stored at higher temperatures, as long as it is kept below its transition temperature (Tg), which is primarily determined by the sugar used. The sugar glass is thought to confer stability to the vaccine due to immobilization, the replacement of water molecules and their hydrogen bonds by sugar molecules, and by creating a physical barrier between two molecules.

Outline of the thesis

The first aim of the thesis is to identify the basic vaccine formulation which is most fit for pandemic use, primarily in terms of immunogenicity, and to unravel its immunogenic mechanisms. Such knowledge may be used for the rational development of improved influenza vaccines for future pandemics and epidemics. The second aim is to further increase vaccine immunogenicity, by use of a conventional adjuvant, and to increase vaccine stability using sugar glass technology.

We first compared the immune responses to whole inactivated virus vaccine (WIV), subunit vaccine, and a virosomal vaccine in a mouse model, using a H3N2 vaccine virus **(Chapter 2)**. Quantitative as well as qualitative differences in the antibody response, T helper cell activation and cytokine production were assessed in mice with different genetic backgrounds. Irrespective of the genetic background of the recipient, WIV induced high levels of Th-1 type antibodies and performed better than subunit or the virosomal vaccine, which correlated with the capacity to induce IFNα production by pDC, and proinflammatory cytokines by cDC *in vitro*.

Because viral RNA, present in WIV but not in subunit or virosomal vaccine, activates TLR7 leading to IFNα production, we assessed the role of TLRs in the adaptive response to different vaccine formulations **(Chapter 3)**. To this end, TLR7 deficient and MyD88/Trif deficient mice were immunized with H5N1 WIV, split-virus or subunit vaccine. TLR7 activation by WIV turned out to play a major role in the differential immune responses exerted by these vaccines.

It has been suggested by others that viral membrane fusion may be important for TLR7 signalling. Fusion activity may therefore be a feature that needs to be preserved in WIV preparation, as it might influence WIV induced immunity. To test this hypothesis we immunized mice with fusion-active and fusion-inactive H5N1 WIV **(Chapter 4)**. Our data shows that fusion activity does not seem to play a major role in the antibody response to WIV.

The use of WIV vaccines and vaccine/adjuvant combinations have both been suggested as dose-sparing strategies. We investigated the combination of these two strategies by immunizing mice with Aluminium hydroxide (Alum)-adjuvanted H1N1 WIV **(Chapter 5)**. Alum had a clear adjuvant effect on the quantity of the antibody response to WIV, but changed the induced immune phenotype. Remarkably, in a virus challenge experiment the addition of Alum to WIV had a deteriorating effect on the protective immunity induced by vaccination.

The quality of the vaccine-induced immune response in terms of a Th1- or Th2-phenotype appears to be an important issue in vaccine development.

Last, we studied the stability of pre-pandemic H5N1 WIV by assessing its antibody-inducing property after storage at different temperatures **(Chapter 6)**. We show that WIV in suspension is stable for at least one year at room temperature. However, storage at higher temperatures requires stabilization. We tested sugarglass technology to stabilize WIV, using trehalose and inulin as stabilizing sugars.

The results of these studies are further discussed in **Chapter 7** and **Chapter 8.**

References

1. Glezen WP. Emerging infections: pandemic influenza. Epidemiol Rev. 1996;18(1): 64-76.
2. Potter CW. A history of influenza. J Appl Microbiol. 2001;91(4):572-9.
3. Neumann G, Kawaoka Y. The first influenza pandemic of the new millennium. Influenza Other Respi Viruses. 2011;5(3):157-66.
4. Potter CW, Jennings R. A definition for influenza pandemics based on historial records. J Infect. 2011;63(4):252-9.
5. Hilleman MR. Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. Vaccine. 2002;20(25-26):3068-87.
6. [6a] http://www.who.int/mediacentre/factsheets/2003/fs211/en/ [6b] Estimates of Deaths Associated with Seasonal Influenza --- United States, 1976—2007.CDC MMWR. 2010;59(33);1057-1062 http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5933a1.htm
7. http://www.who.int/csr/don/2010 05 14/en/index.html
8. Viboud C, Miller M, Olson D, Osterholm M, Simonsen L. Preliminary Estimates of Mortality and Years of Life Lost Associated with the 2009 A/H1N1 Pandemic in the US and Comparison with Past Influenza Seasons. PLoS Curr. 2010:RRN1153.
9. Johnson NP, Mueller J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med. 2002;76(1):105-15.
10. Webby RJ, Webster RG. Are we ready for pandemic influenza? Science. 2003;302(5650):1519-22.
11. Palese P, Shaw ML. Orthomyxoviridae: The Viruses and Their Replication. In: Knipe DM, Howley PM, editors. Fields Virology. 5th Edition. Vol. 2. Philadelphia: Lippincott Williams & Wilkins; 2007. pp. 1647–1689.
12. Sambhara S, Poland GA. H5N1 Avian influenza: preventive and therapeutic strategies against a pandemic. Annu Rev Med. 2010;61:187-98.
13. Lanzrein M, Schlegel A, Kempf C. Entry and uncoating of enveloped viruses. Biochem J. 1994 ;302 (Pt 2):313-20.
14. Lakadamyali M, Rust MJ, Zhuang X. Endocytosis of influenza viruses. Microbes Infect. 2004;6(10):929-36.
15. Kilbourne ED. Influenza pandemics of the 20th century. Emerg Infect Dis. 2006;12(1):9-14.
16. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009;360(25):2605-15.

17. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992;56(1):152-79.

18. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol. 2005;79(5):2814-22.

19. Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. Science. 2006;312(5772):384-8.

20. Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol. 1989;63(11):4603-8.

21. Webster RG, Laver WG. The origin of pandemic influenza. Bull World Health Organ. 1972;47(4):449-52.

22. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, López-Gatell H, Olivera H, López I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD Jr, Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 2009;325(5937):197-201.

23. Smith W, Andrewes CH, Laidlaw PP. A virus obtained from influenza patients. Lancet 1933;2:66-8.

24. Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, Fanning TG. Initial genetic characterization of the 1918 "Spanish" influenza virus. Science. 1997;275(5307): 1793-6.

25. Reid AH, Fanning TG, Hultin JV, Taubenberger JK. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. Proc Natl Acad Sci U S A. 1999;96(4):1651-6.

26. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. Nature. 2005;437(7060):889-93.

27. Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, Chen H, Webster RG, Peiris JS, Guan Y. Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci U S A. 2009;106(28):11709-12.

28. Guan Y, Vijaykrishna D, Bahl J, Zhu H, Wang J, Smith GJ. The emergence of pandemic influenza viruses. Protein Cell. 2010;1(1):9-13.

29. Palese P, García-Sastre A. Influenza vaccines: present and future. J Clin Invest. 2002;110(1):9-13.

30. Dormitzer PR, Galli G, Castellino F, Golding H, Khurana S, Del Giudice G, Rappuoli R. Influenza vaccine immunology. Immunol Rev. 2011;239(1):167-77.

31. Hayden EC. Avian influenza aided readiness for swine flu. Nature. 2009;459(7248): 756-7.

32. Abelin A, Colegate T, Gardner S, Hehme N, Palache A. Lessons from pandemic influenza A(H1N1): the research-based vaccine industry's perspective. Vaccine. 2011;29(6):1135-8.

33. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet. 1998;351(9101):472-7.

34. Tam JS. Influenza A (H5N1) in Hong Kong: an overview. Vaccine. 2002;20 Suppl 2:S77-81.

35. http://www.who.int/csr/disease/avian influenza/2010 08 31 h5n1 avian influenza timeline updates.pdf

36. Chan PK. Outbreak of avian influenza A(H5N1) virus infection in Hong Kong in 1997. Clin Infect Dis. 2002;34 Suppl 2:S58-64.

37. Webster RG. Wet markets--a continuing source of severe acute respiratory syndrome and influenza? Lancet. 2004;363(9404):234-6.

38. http://apps.who.int/gb/ebwha/pdf files/EB115/B115 44-en.pdf

39. http://www.who.int/csr/disease/avian influenza/country/cases table 2011 06 22/en/index.html

40. Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, Pham TS, Vo CD, Le TQ, Ngo TT, Dao BK, Le PP, Nguyen TT, Hoang TL, Cao VT, Le TG, Nguyen DT, Le HN, Nguyen KT, Le HS, Le VT, Christiane D, Tran TT, Menno de J, Schultsz C, Cheng P, Lim W, Horby P, Farrar J; World Health Organization International Avian Influenza Investigative Team. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med. 2004;350(12):1179-88.

41. Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, Uiprasertkul M, Boonnak K, Pittayawonganon C, Cox NJ, Zaki SR, Thawatsupha P, Chittaganpitch M, Khontong R, Simmerman JM, Chunsutthiwat S. Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med. 2005;352(4):333-40.

42. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Avian flu: influenza virus receptors in the human airway. Nature. 2006;440(7083):435-6.

43. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T. H5N1 Virus Attachment to Lower Respiratory Tract. Science. 2006;312(5772):399.

44. Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science. 2006;312(5772):404-10.

45. [45a]Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog. 2007;3(10):1374-9. [45b]http://www.newscientist.com/article/mg21128314.600-five-easy-mutations-to-make-bird-flu-a-lethal-pandemic.html

46. http://www.who.int/csr/resources/publications/influenza/WHO CDS CSR GIP 2005 7 04.pdf

47. The Writing Committee of the World Health Organization (WHO) Consultation on Human Influenza A/H5. Avian influenza A (H5N1) infection in humans. N Engl J Med 2005;353:1374-85.

48. Gambotto A, Barratt-Boyes SM, de Jong MD, Neumann G, Kawaoka Y. Human infection with highly pathogenic H5N1 influenza virus. Lancet. 2008;371(9622): 1464-75.

49. Lipatov AS, Govorkova EA, Webby RJ, Ozaki H, Peiris M, Guan Y, Poon L, Webster RG. Influenza: emergence and control. J Virol. 2004;78(17):8951-9.

50. Korteweg C, Gu J. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. Am J Pathol. 2008;172(5):1155-70.

51. Zhang Z, Zhang J, Huang K, Li KS, Yuen KY, Guan Y, Chen H, Ng WF. Systemic infection of avian influenza A virus H5N1 subtype in humans. Hum Pathol. 2009;40(5):735-9.

52. Schrauwen EJ, Bestebroer TM, Munster VJ, de Wit E, Herfst S, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. Insertion of a multibasic cleavage site in the haemagglutinin of human influenza H3N2 virus does not increase pathogenicity in ferrets. J Gen Virol. 2011;92(Pt 6):1410-5.

53. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, Gordon S, Guan Y, Peiris JS. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet. 2002;360(9348):1831-7.

54. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha do Q, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med. 2006;12(10): 1203-7.

55. http://www.who.int/csr/disease/avian influenza/phase/en/

56. Fedson DS. Vaccine development for an imminent pandemic: why we should worry, what we must do. Hum Vaccin. 2006;2(1):38-42.

57. http://www.who.int/csr/resources/publications/influenza/WHO CDS CSR GIP 2005 5/en/

58. Uscher-Pines L, Omer SB, Barnett DJ, Burke TA, Balicer RD. Priority setting for pandemic influenza: an analysis of national preparedness plans. PLoS Med. 2006;3(10):e436.

59. Monto AS. Vaccines and antiviral drugs in pandemic preparedness. Emerg Infect Dis. 2006;12(1):55-60.

60. Leneva IA, Roberts N, Govorkova EA, Goloubeva OG, Webster RG. The neuraminidase inhibitor GS4104 (oseltamivir phosphate) is efficacious against A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) influenza viruses. Antiviral Res. 2000;48(2):101-15.

61. Govorkova EA, Leneva IA, Goloubeva OG, Bush K, Webster RG. Comparison of efficacies of RWJ-270201, zanamivir, and oseltamivir against H5N1, H9N2, and other avian influenza viruses. Antimicrob Agents Chemother. 2001;45(10):2723-32.

62. Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus, Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soeroso S, Uyeki TM. Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med. 2008;358(3):261-73.

63. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J Virol. 2004;78(22):12665-7.

64. Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, Pham ND, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y. Avian flu: isolation of drug-resistant H5N1 virus. Nature. 2005;437(7062):1108.

65. de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, Nguyen VC, Bach VC, Phan TQ, Do QH, Guan Y, Peiris JS, Tran TH, Farrar J. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med. 2005;353(25):2667-72.

66. Moscona A. Oseltamivir resistance--disabling our influenza defenses. N Engl J Med. 2005;353(25):2633-6.

67. Hayden FG, Pavia AT. Antiviral management of seasonal and pandemic influenza. J Infect Dis. 2006;194 Suppl 2:S119-26.

68. http://www.who.int/csr/disease/avian influenza/final.pdf

69. Horimoto T, Kawaoka Y. Strategies for developing vaccines against H5N1 influenza A viruses. Trends Mol Med. 2006;12(11):506-14.

70. [70] Kreijtz JH, Osterhaus AD, Rimmelzwaan GF. Vaccination strategies and vaccine formulations for epidemic and pandemic influenza control. Hum Vaccin. 2009;5(3):126-35.

71. Bridges CB, Katz J, Levandowski R, Cox NJ. Inactivated influenza vaccines. In: Plotkin SA, Orenstein WA, Offit PA, eds. Vaccines 5th Edition: Saunders Elsevier 2008; 259-90.

72. Stegmann T, Morselt HW, Booy FP, van Breemen JF, Scherphof G, Wilschut J. Functional reconstitution of influenza virus envelopes. EMBO J 1987;6:2651–2659.

73. Dormitzer PR, Galli G, Castellino F, Golding H, Khurana S, Del Giudice G, Rappuoli R. Influenza vaccine immunology. Immunol Rev. 2011;239(1):167-77.

74. de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaus AD. Haemagglutination-inhibiting antibody to influenza virus. Dev Biol (Basel). 2003;115:63-73.

75. Rimmelzwaan GF, Fouchier RA, Osterhaus AD. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. Curr Opin Biotechnol. 2007;18(6):529-36.

76. Longini IM Jr, Nizam A, Xu S, Ungchusak K, Hanshaoworakul W, Cummings DA, Halloran ME. Containing pandemic influenza at the source. Science. 2005;309(5737):1083-7.

77. Ferguson NM, Cummings DA, Cauchemez S, Fraser C, Riley S, Meeyai A, Iamsirithaworn S, Burke DS. Strategies for containing an emerging influenza pandemic in Southeast Asia. Nature. 2005;437(7056):209-14.

78. http://www.who.int/csr/disease/avian influenza/guidelines/RapidContProtOct15.pdf

79. Jennings LC, Monto AS, Chan PK, Szucs TD, Nicholson KG. Stockpiling prepandemic influenza vaccines: a new cornerstone of pandemic preparedness plans. Lancet Infect Dis. 2008;8(10):650-8.

80. Swerdlow DL, Finelli L, Bridges CB. 2009 H1N1 influenza pandemic: field and epidemiologic investigations in the United States at the start of the first pandemic of the 21st century. Clin Infect Dis. 2011;52 Suppl 1:S1-3.

81. Watson JM, Pebody RG. Pandemic influenza vaccines. BMJ. 2011;342:d545.

82. Monto AS, Black S, Plotkin SA, Orenstein WA. Response to the 2009 pandemic: Effect on influenza control in wealthy and poor countries. Vaccine. 2011;29(38): 6427-31.

83. http://www.who.int/csr/disease/influenza/A58 13-en.pdf

84. Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med. 2006;354(13):1343-51.

85. Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, McClain-Moss LR, Peiris JS, Rehg JE, Tuomanen EI, Webster RG. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet. 2004;363(9415):1099-103.

86. I. Leroux-Roels, A. Borkowski, T. Vanwolleghem, M. Drame, F. Clement, E. Hons, J.M. Devaster, and G. Leroux-Roels. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet. 2007;370(9587):580-9.

87. K. Levie, I. Leroux-Roels, K. Hoppenbrouwers, A.D. Kervyn, C. Vandermeulen, S. Forgus, G. Leroux-Roels, S. Pichon, and I. Kusters. An adjuvanted, low-dose,

pandemic influenza A (H5N1) vaccine candidate is safe, immunogenic, and induces cross-reactive immune responses in healthy adults. J Infect Dis. 2008;198(5):642-9.

88. J. Lin, J. Zhang, X. Dong, H. Fang, J. Chen, N. Su, Q. Gao, Z. Zhang, Y. Liu, Z. Wang, M. Yang, R. Sun, C. Li, S. Lin, M. Ji, X. Wang, J. Wood, Z. Feng, Y. Wang, and W. Yin. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet. 2006;368(9540):991-7.
89. Chua JV, Chen WH. Bench-to-bedside review: vaccine protection strategies during pandemic flu outbreaks. Crit Care. 2010;14(2):218.
90. Nichol KL. Efficacy/clinical effectiveness of inactivated influenza virus vaccines in adults. In: Nicholson KG, Webster RG, Hay AJ, eds. Textbook on influenza: Oxford: Blackwell Science 1998; 358-72.
91. Beigel JH, Voell J, Huang CY, Burbelo PD, Lane HC. Safety and immunogenicity of multiple and higher doses of an inactivated influenza A/H5N1 vaccine. J Infect Dis. 2009;200(4):501-9.
92. Nicholson KG, Tyrrell DA, Harrison P, Potter CW, Jennings R, et al. Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: Serological responses and clinical reactions. J Biol Stand. 1979;7(2):123-36.
93. Beyer WE, Palache AM, Osterhaus AD. Comparison of serology and reactogenicity between influenza subunit vaccines and whole virus or split vaccines: A review and meta-analysis of the literature. Clin Drug Investig. 1998;15(1):1-12.
94. Stephenson I, Nicholson KG, Glück R, Mischler R, Newman RW, et al. Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: Phase I randomised trial. Lancet. 2003;362(9400):1959-66.
95. Greenberg ME, Lai MH, Hartel GF, Wichems CH, Gittleson C, Bennet J, Dawson G, Hu W, Leggio C, Washington D, Basser RL. Response to a monovalent 2009 influenza A (H1N1) vaccine. N Engl J Med. 2009;361(25):2405-13.
96. Centers for Disease Control and Prevention (CDC). Update on influenza A (H1N1) 2009 monovalent vaccines. MMWR Morb Mortal Wkly Rep. 2009;58(39):110-1.
97. Goji NA, Nolan C, Hill H, Wolff M, Noah DL, Williams TB, Rowe T, Treanor JJ. Immune responses of healthy subjects to a single dose of intramuscular inactivated influenza A/Vietnam/1203/2004 (H5N1) vaccine after priming with an antigenic variant. J Infect Dis. 2008;198(5):635-41.
98. Ehrlich HJ, Muller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N Engl J Med. 2008;358:2573–84.
99. Baras B, Stittelaar KJ, Simon JH, Thoolen RJ, Mossman SP, Pistoor FH, et al. Cross-protection against lethal H5N1 challenge in ferrets with an adjuvanted pandemic influenza vaccine. PLoS One. 2008;3:e1401.

100.Fazekas G, Martosne-Mendi R, Jankovics I, Szilvasy I, Vajo Z. Cross-reactive immunity to clade 2 strains of influenza virus A subtype H5N1 induced in adults and elderly patients by Fluval, a prototype pandemic influenza virus vaccine derived by reverse genetics, formulated with a phosphate adjuvant, and directed to clade 1 strains. Clin Vaccine Immunol. 2009;16:437–43.

101.Govorkova EA, Webby RJ, Humberd J, Seiler JP, Webster RG. Immunization with reverse-genetics-produced H5N1 influenza vaccine protects ferrets against homologous and heterologous challenge. J Infect Dis. 2006;194:159–67.

102.Murakami S, Iwasa A, Iwatsuki-Horimoto K, Ito M, Kiso M, Kida H, et al. Cross-clade protective immunity of H5N1 influenza vaccines in a mouse model. Vaccine. 2008;26:6398–404.

103.Julkunen I, Hovi T, Seppälä I, Mäkelä O. Immunoglobulin G subclass antibody responses in influenza A and parainfluenza type 1 virus infections. Clin Exp Immunol. 1985;60(1):130-8.

104.Johnson PR, Feldman S, Thompson JM, Mahoney JD, Wright PF. Immunity to influenza A virus infection in young children: a comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. J Infect Dis. 1986;154(1):121-7.

105.Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA, Makarova N, Perez DR, Macdonald GH, McCullers JA. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol. 2006;13(9):981-90.

106.Hovden AO, Cox RJ, Madhun A, Haaheim LR. Two doses of parenterally administered split influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral shedding upon challenge in mice. Scand J Immunol. 2005;62(4):342-52.

107.Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J Virol. 1996;70(5):2852-60.

108.Netea MG, Van der Meer JW, Sutmuller RP, Adema GJ, Kullberg BJ. From the Th1/Th2 paradigm towards a Toll-like receptor/T-helper bias. Antimicrob Agents Chemother. 2005;49(10):3991-6.

109.Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.

110.Diebold SS. Recognition of viral single-stranded RNA by Toll-like receptors. Adv Drug Deliv Rev. 2008;60(7):813-23.

111.Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, Kopf M, Marsland BJ. TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. J Immunol. 2007;178(4):2182-91.

112.Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int J Pharm. 1999;185(2):129-88.

113.Coenen F, Tolboom JT, Frijlink HW. Stability of influenza sub-unit vaccine. Does a couple of days outside the refrigerator matter? Vaccine. 2006;24(4):525-31.

114. Amorij JP, Hinrichs WLj, Frijlink HW, Wilschut JC, Huckriede A. Needle-free influenza vaccination. Lancet Infect Dis. 2010;10(10):699-711.

115.Saluja V, Hinrichs WL, Frijlink HW. Dried influenza vaccines: over the counter vaccines. Hum Vaccin. 2010;6(10):854-6.

116.Huang J, Garmise RJ, Crowder TM, Mar K, Hwang CR, Hickey AJ, et al. A novel dry powder influenza vaccine and intranasal delivery technology: induction of systemic and mucosal immune responses in rats. Vaccine. 2004;23:794–801.

117.Singh M, Briones M, O'Hagan DT. A novel bioadhesive intranasal delivery system for inactivated influenza vaccines. J Control Release. 2001;70(3):267-76.

118.Amorij JP, Meulenaar J, Hinrichs WL, Stegmann T, Huckriede A, Coenen F, Frijlink HW. Rational design of an influenza subunit vaccine powder with sugar glass technology: preventing conformational changes of haemagglutinin during freezing and freeze-drying. Vaccine. 2007;25(35):6447-57.

119.de Jonge J, Amorij JP, Hinrichs WL, Wilschut J, Huckriede A, Frijlink HW. Inulin sugar glasses preserve the structural integrity and biological activity of influenza virosomes during freeze-drying and storage. Eur J Pharm Sci. 2007;32(1):33-44.

120.Amorij JP, Huckriede A, Wilschut J, Frijlink HW, Hinrichs WL. Development of stable influenza vaccine powder formulations: challenges and possibilities. Pharm Res. 2008;25(6):1256-73.

Whole inactivated virus influenza vaccine is superior to subunit vaccine in inducing immune responses and secretion of proinflammatory cytokines by DCs

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Abstract

Background: For protection against (re-)infection by influenza virus not only the magnitude of the immune response but also its quality in terms of antibody subclass and T helper profile are important. Information about the type of immune response elicited by vaccination is therefore urgently needed.

Objectives: This study aims at evaluating in detail the immune response elicited by three current influenza vaccine formulations and at shedding light on vaccine characteristics which determine this response.

Methods: Mice were immunized with whole inactivated virus (WIV), virosomes (VS), or subunit vaccine (SU). Following subsequent infection with live virus, serum antibody titers and Th cell responses were measured. The effects of the vaccines on cytokine production by conventional and plasmacytoid dendritic cells were investigated in vitro.

Results and Conclusions. In Balb/c mice (Th2 prone) as well as in C57Bl/6 mice (Th1 prone) WIV induced consistently higher hemagglutination-inhibition titers and virus-neutralizing antibody titers than VS or SU. In contrast to VS and SU, WIV stimulated the production of the antibody subclass IgG2a (Balb/c) and IgG2c (C57BL/6) considered to be particularly important for viral clearance and activation of IFNγ producing T cells. Similar to live virus WIV stimulated the production of proinflammatory cytokines by conventional dendritic cells and IFNα by plasmacytoid cells while VS and SU had little effect on cytokine synthesis by either cell type. We conclude that vaccination with WIV in contrast to VS or SU results in the desired Th1 response presumably by induction of type I interferon and other proinflammatory cytokines.

Introduction

Influenza remains one of the major infectious diseases with 3 to 5 million severe cases of illness and 250-500.000 deaths per year in industrialized countries only according to estimates of the World Health Organization [1]. Next to the yearly epidemics there is the eminent threat of a new influenza pandemic with an estimated attack rate of 10-30% and possibly a very high death toll [2,3]. Vaccination has been and will be the cornerstone of influenza control in epidemic and pandemic situations.

Among the available vaccine formulations split and subunit vaccines are most frequently used for immunization against the yearly influenza epidemics [4]. Split vaccines consist of inactivated virus particles which are disrupted by treatment with detergent and/or ether. Subunit vaccines (SU) consist of the viral surface antigens purified from detergent-disrupted virus particles. Virosomal vaccines (VS) which have been introduced on the market recently are reconstituted viral membranes consisting of the viral surface antigens inserted in a lipid bilayer thus mimicking the viral envelope [5,6]. In the early years of influenza vaccination, whole inactivated virus (WIV) has also been used as influenza vaccine. However, the use of WIV vaccines was largely abandoned due to a higher incidence of side effects as compared to the other formulations. Recently, WIV has regained interest in the context of pandemic vaccine development as a simple and highly immunogenic vaccine formulation. Reactogenicity of modern WIV appears to be comparable to other vaccine formulations possibly due to improved methods for virus production and purification [7-11].

For the evaluation of vaccine efficacy the serum hemagglutination-inhibition (HI) titer achieved by vaccination is currently used as the only correlate of protection. An HI titer of 40 is estimated to be associated with a 50% reduction of the risk of contracting influenza and is used as the basis for the EMEA criteria to which influenza vaccines have to comply [4]. This 50% protective titer was calculated from a number of clinical studies in which immunity was achieved by either natural infection or by vaccination with inactivated or live-attenuated influenza vaccines. Since infection as well as vaccination will induce a plethora of immune reactions it is unclear whether HI antibodies themselves provide protection or whether their presence is simply an indication for the immune status to influenza virus [12].

Recent evidence suggests that in addition to the magnitude of the immune reaction also the quality of the immune response is important for protection. The antibody response to natural infection by influenza virus and other viruses in mice is dominated by IgG2a (or in C57BL/6 mice IgG2c) [13-16]. By virtue of its Fc domain murine IgG2a/2c interacts very efficiently with complement factors and activatory Fc receptors [17-19]. Thereby, IgG2a/2c contributes to viral clearance by activation of the complement system, stimulation of antibody dependent cellular cytotoxicity and clearance of opsonized virus by macrophages [16, 20,21]. In contrast, IgG1 does neither activate complement efficiently nor has it a high affinity for activatory Fc receptors [18,19]. Recently, it was shown that IgG2a alone protects mice as efficiently from lethal challenge as a mixture of IgG1 and IgG2a. In contrast, IgG1 alone only protects from mild virus challenge but provides insufficient protection upon high dose challenge [22].

The relative contribution of Th1 and Th2 cell-mediated effector mechanisms to protection from influenza virus-induced lung damage and to virus clearance is still under investigation. T helper cell responses to influenza infection involve IFNγ producing Th1 cells as well as IL4 producing Th2 cells but Th1 responses are strongly dominant [23]. Evidence is accumulating that Th1 cells are superior to Th2 cells in providing protection against viral infection and do so by secretion of IFNγ and by stimulation of B cells and $CD8^+$ T cells but also by direct perforin-dependent cytolysis [24-29]. On the other hand, Th2 cells are necessary to prevent excessive lung inflammation caused by an overwhelming Th1 response [27]. In the absence of antibodies, T helper cells can provide a certain degree of cross-protective immunity possibly by secretion of IFNγ which activates macrophages or by direct cytolysis of infected cells [25,26,29]. Since epitopes recognized by T helper cells are more conserved than those recognized by antibodies vaccine-induced T helper responses might contribute decisively to the cross-protective potential of influenza vaccines [29].

In order to shed more light on the quality of the immune response to different influenza vaccine formulations we immunized mice with WIV, VS or SU vaccines and measured the induced HI titers, virus-neutralizing antibody titers, virus specific IgG1 and IgG2a/2c and determined the Th1/Th2 balance by enumeration of IFNγ and IL4 producing T helper cells. Since the genetic background of mice is known to have a large effect on the immune response, the vaccination experiments were performed in Th1 prone C57BL/6 mice as well as in Th2 prone Balb/c mice. To obtain insight into the mechanisms responsible for the

differential reactions to the vaccines we studied the effect of the vaccine formulations on conventional and plasmacytoid dendritic cells *in vitro*. These cell types are considered as the most important switches between the innate and the adaptive immune system and are essential for the induction and control of specific immune responses. Our results show that in contrast to VS and SU vaccine, WIV induces a strong immune response qualitatively resembling the response obtained after virus infection irrespective of the genetic background of the recipient. The immune response to the vaccines *in vivo* is likely to be related to their capability to induce the production of proinflammatory cytokines by conventional DCs and IFNα by plasmacytoid DCs.

Material & Methods

MICE

Ten to twelve week old female Balb/c mice or C57BL/6 mice, purchased from Harlan Netherlands B.V. (Zeist, The Netherlands), were used for the immunization study or alternatively for isolation of spleen cells and bone marrow cells for *in vitro* stimulation experiments. Animal experiments were conducted according to the guidelines provided by the Dutch Animal Protection Act, and were approved by the Committee for Animal Experimentation (DEC) of the University of Groningen.

VIRUSES AND VACCINES

Egg-derived A/Panama/2007/99 (H3N2) virus and subunit vaccine produced from this strain were a kind gift from Solvay Pharmaceuticals, Weesp, The Netherlands.

Whole inactivated virus vaccine (WIV) was produced by inactivation of virus with 0.1% β-propiolactone (Acros Organics, Geel, Belgium) for 24 hours at 19-21 °C, followed by dialysis and filtration (0.45 μm). Virosomes were produced as described previously [5,6]. In short, membrane lipids of β-propiolactone inactivated virus were solubilized with detergent octa (ethyleneglycol)-*n*-dodecyl monoether ($C_{12}E_8$) (Nikkol, Tokyo, Japan). Nucleocapsids were removed by ultracentrifugation and membranes were reconstituted by extraction of $C_{12}E_8$ using Biobeads (Bio-Rad, Hercules, CA). So formed virosomes were concentrated by ultracentrifugation on a 50 % sucrose cushion in Hepes buffered saline/ EDTA buffer (HNE), followed by dialysis and filtration.

IMMUNIZATIONS AND INFECTIONS

Mice were infected intranasally with 150 hemagglutination units (HAU) live A/Panama virus or were intramuscularly immunized in the hind leg with 30 µl of the different vaccine formulations each containing 5 µg viral hemagglutinin protein (HA) in HNE buffer or received buffer only. After 28 days serum samples were collected prior to an intranasal boost with 150 HAU live influenza virus in 10 µl divided over both nostrils. Three days hereafter mice were bled to death and spleens were collected for T helper cell evaluation.

HEMAGGLUTINATION-INHIBITION ASSAY

A standard HAI assay was performed. In short, 75 µl serum was inactivated at 56 °C for 30 min and absorbed to 225 µl 25 % kaolin/PBS (Sigma-Aldrich, Inc., St. Louis, MO) solution for 20 min at room temperature (RT). After centrifugation 50 µl supernatant was added to 50 µl PBS in duplicate in a round-bottom microtiter plate (Costar, Corning Inc., Corning, NY) followed by two-fold serial dilutions. 4 hemagglutination units (HAU) of virus in 50 µl PBS were added to each well and the mixtures were incubated for 40 min at RT. Finally, 50 µl of 1% guinea pig erythrocytes (Harlan) in PBS was added to each well and HAI titers were determined after 2 hours incubation at RT. HAI titers represent the reciprocal of the highest serum dilution yielding complete inhibition of hemagglutination. HAI titers below the detection limit were assigned with half the value of the lowest serum dilution.

VIRUS-NEUTRALIZATION ASSAY

Virus-neutralizing (VN) serum antibodies were assessed by a VN assay described previously [30]. Briefly, quadruplicates of two-fold serum dilutions in (serum free) cell culture medium were incubated with an equal volume containing 6.25 TCID50 virus, at 37 °C for 2 hours before 100 µl of this mix was added to Maden Darby canine kidney (MDCK) cell monolayers in a microtiter plate (Costar, Corning Inc.). After overnight incubation in a humidified CO_2 incubator at 37 °C, cells were fixed with 80% acetone in PBS and the amount of intracellularly produced viral nucleoprotein (NP) was determined by ELISA. Blocking was performed with 150 µl of 4% BSA (Sigma-Aldrich) in 0.05% Tween 20/PBS (PBS/T), 45 min at RT, followed by washing with PBS/T. Subsequently, 100 µl of anti-NP monoclonal antibody (mAb) (Instruchemie, Delfzijl, The Netherlands), diluted 1:8000 in

1%BSA/0.1%Tween 20/PBS, was added for 1 hour at RT, followed by washing. Bound anti-NP mAb was detected by incubation with 100 µl goat anti-mouse IgG-horseradish peroxidase conjugate (Southern Biotech, Birmingham, Alabama), diluted 1:8000 in 1% BSA/0.1%Tween/PBS, 1 hour at RT, followed by washing and subsequent staining with o-phenylene-diamine-dihydrochloride (OPD) (Eastman Kodak Company, Rochester, NY). Absorbance at 492 nm (A_{492}) was measured with an ELISA reader (Bio-tek Instruments, inc., Winooski, VT). VN titers were expressed as the reciprocal of the highest dilution yielding an average A_{492} above the end-point value resulting from the equation: [(average A_{492} of the positive controls (infected cells) minus average A_{492} of the negative controls (non infected cells)) divided by 2] plus the average A_{492} of the negative controls.

ISOTYPE ELISA

For detection of virus specific serum antibodies of different isotypes microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 0.2 µg influenza subunit vaccine in 100 µl 0.05 M carbonate-bicarbonate coating buffer (pH 9.6-9.8) per well, overnight at 37 °C, followed by blocking with 2% milk in coating buffer for 45 minutes at 37°C. After washing with coating buffer and 0.05%Tween 20/PBS (PBS/T), 100 µl of serum diluted in PBS/T was applied in duplicate to the first well and serial twofold dilutions were made. A subsequent incubation for 1.5 hours at 37°C was followed by washing and incubation with 100 µl of horseradish peroxidase conjugated goat anti-mouse IgG-isotype antibody (Southern Biotech) for 1 hour at 37°C. Plates were washed and stained with OPD (Eastman Kodak Company). Absorbance at 492 nm (A_{492}) was read with an ELISA reader (Bio-tek Instruments, inc.). After subtraction of background levels, serum antibody concentrations were calculated by means of appropriate isotype standards (Southern Biotech) using linear regression.

ELISPOT

IFNγ and IL4 ELISPOT asays were performed as described before [31] with some adaptations. In short, erythrocyte-depleted splenocytes ($5x10^5$ cells per 100 µl 5% FCS/50 µM β-mercaptoethanol/IMDM medium (Gibco, Paisley, UK)) were seeded in triplicate on a microtiter plate (Greiner), pre-coated with anti-IFNγ or anti-IL4 capture antibodies (Pharmingen, San Diego, CA) and blocked with 4% BSA/PBS (Sigma-Aldrich). Cells were stimulated with 1 µg A/Panama virosomes per well, overnight in a humidified CO_2 incubator at 37 °C. Plates were treated

with 100 µl of H_2O per well and kept on ice to lyse the cells. After washing with 0.02% Tween 20/PBS, biotinylated anti-IFNγ or anti-IL4 antibody (Pharmingen) was added at a concentration of 0.125 µg/ ml in 2% BSA/PBS (Sigma-Aldrich), 50 µl/ well, and incubated for 1 hour at 37 °C. After washing and incubation with alkaline phosphatase conjugated streptavidin (Pharmingen), 1:1000 diluted in 2% BSA/PBS, 100 µl/ well for 1 hr at 37°C, spots were visualized with a 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich) substrate reaction immobilized in solidified agarose. Plates were scanned and spots were counted manually.

Culture of conventional DCs

Femurs from Balb/c mice were dissected and flushed with IMDM (Gibco) to collect bone marrow (BM). BM leukocytes were seeded at $2x10^6$ cells in a 100 mm bacteriological petri dish (Corning) in the presence of 200 U/ml recombinant mouse (rm) GM-CSF (Peprotech, London, UK) as described in detail by Lutz and colleagues [32].

After 9 days of culture, the non-adherent cells were collected by gentle pipetting, and centrifugation at 300 g for 5 minutes at RT. FACS analysis showed over 70% of these cells to be CD11c positive, representing cDCs. $1.5x10^7$ cells were seeded per culture dish (100 mm, Corning) in 10 ml fresh medium containing 100 U/ml rmGM-CSF. To induce maturation and cytokine production, cDCs were exposed to active virus with a multiplicity of infection (MOI) of 1.5, or to different inactivated vaccine formulations (10 µg HA per ml). After 4, 12 and 24 hours incubation in a humidified CO_2 incubator at 37 °C supernatants were collected for cytokine quantification by Luminex multiplex immunoassay.

Luminex assay

The multiplex technology (Luminex Corp., Oosterhout, The Netherlands) combines the principle of a sandwich immunoassay with fluorescent bead–based technology, allowing individual and multiplex analysis of up to 100 different analytes in a single microtiter well [33]. The multiplex assay for 6 cytokines [IL1β, IL6, IL10, IL12p70, TNFα, IFNγ] was performed in 96-well microtiter plate format according to the manufacturers protocol (LINCO Research, Inc, Missouri, USA). Samples were analyzed on a Luminex 100 apparatus, and calculations were performed using STarStation software (Applied Cytometry Systems, Sheffield, UK).

PLASMACYTOID DENDRITIC CELLS

Single splenocyte suspensions were produced as described in the ELISPOT section and enriched for plasmacytoid DCs (pDCs) by depletion of T cells, B cells, NK cells and macrophages by magnetically labeling and separating CD3, CD19, CD 11b and CD49b positive cells (Miltenyi Biotech GmbH, Germany). Labeling of pDCs with anti-mPDCA-1-PE antibody (Miltenyi) for FACS analysis, revealed a final pDCs population constituting approximately 6 % of the enriched cell population. Cell suspensions containing 1-2x10^5 pDCs in 100 µl were seeded in a microtiter plate and stimulated in triplicate with an equal volume containing the appropriate amount of vaccine or live virus for 20 hours in a humidified CO_2 incubator at 37 °C. Culture supernatants were collected and subjected to the IFNα ELISA .

IFNα ELISA

Two fold serial dilutions of culture supernatants, starting from a 4 fold primary dilution, were subjected in duplicate to an IFNα ELISA previously described by Lund et al. [34], except that the staining was performed with OPD (Eastman Kodak Company) and absorbance was read at 492 nm. IFNα concentrations were calculated from a recombinant IFNα standard curve performed in quadruplicate (HyCult, Biotechnology, Uden, The Netherlands) using linear regression, and expressed in units per ml.

STATISTICAL ANALYSIS

Statistical analysis on antibody titers was performed using the unpaired Student's t test. P values of $p<0.05$ and $p<0.01$ were considered as statistically significant or highly significant, respectively.

Results

In order to elucidate the effect of vaccine formulation on the magnitude and the quality of the elicited immune response, mice were immunized once with 5 µg HA derived from A/Panama/2007/99 (H3N2) formulated as WIV, VS, or SU vaccine. Serum samples were taken four weeks after immunization and the hemagglutination-inhibiting (HAI) and virus-neutralizing (VN) capacity of the sera was determined (Table 1). In Balb/c mice, all three vaccines induced HAI titers > 40, with WIV producing the highest responses. In C57BL/6 mice, HAI

titers after immunization with WIV were similarly high as in Balb/c mice but were low (<40) after immunization with VS or SU. The neutralizing activities of the sera correlated with the HAI titers. In both mouse strains they were highest after WIV immunization. Immunization with VS or SU vaccine induced measurable virus-neutralizing activity in Balb/c mice, whereas this activity was under the detection limit of 80 in C57BL/6 mice.

Table 1: Hemagglutination-inhibition titers and virus-neutralization titers after a single immunization.

	Balb/c		C57Bl/6	
	HAI	**VN**	**HAI**	**VN**
HNE	<8	<80	<8	<80
WIV	256	691** (577-823)	256	1493** (959-2457)
VS	128	160* (125-200)	16	<80
SU	128	148* (87-234)	16	<80

*Mice were immunized once i.m. with buffer only (HNE) or with 5 μg HA formulated as WIV, VS, or SU. On day 28 serum samples were taken. For HAI determination serum samples were pooled per group (n=9). For determination of VN titers sera from individual mice were tested. Titer is given as geometric mean, the 95% confidence interval is indicated. Statistical significant differences (P<0.01) are as indicated: ** compared to VS, SU or HNE, * compared to HNE.*

Twenty-eight days after immunization the mice were infected intranasally with live A/Panama virus and early memory responses were determined three days later. Infection with A/Panama virus does not lead to symptomatic disease in mice but induces nevertheless humoral and cellular immune responses. Intranasal administration of live A/Panama virus boosted the vaccine-induced HAI and VN titers in either mouse strain (Fig. 1). In Balb/c mice immunization with all three vaccines resulted in high HAI titers and VN titers after virus exposure (Fig. 1, left panels). Yet, titers in WIV-immunized mice were higher than in VS- and SU-immunized animals. These differences were statistically significant for HAI titers after WIV- vs SU immunization ($p<0.05$) and for VN titers compared between WIV and VS as well as SU ($p<0.01$). In C57BL/6 mice only WIV induced consistently high HAI and VN titers in all immunized mice whereas VS or SU resulted in measurable HAI and VN titers in only some of the immunized animals (Fig. 1, right panels). Differences in titer between WIV- and VS- or SU-immunized mice were highly significant ($p<0.01$) in all cases. Taken together these results show that WIV is more immunogenic than VS or SU irrespective of the mouse strain studied.

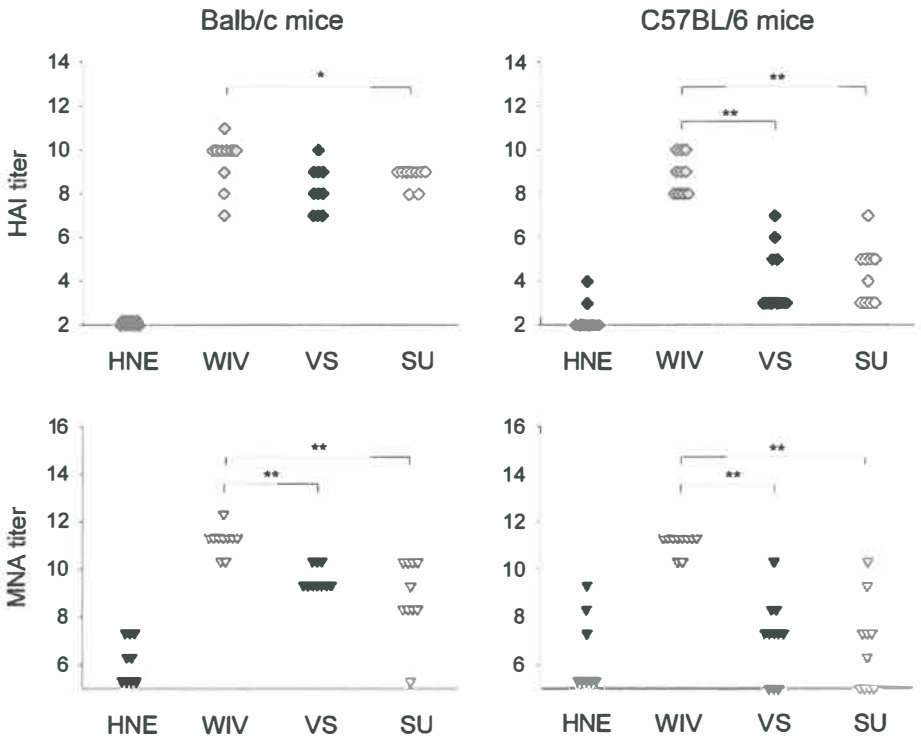

Figure 1: HAI titers and VN titers after immunization followed by virus challenge.
*Mice (9-10/experimental group) were injected i.m. with buffer (HNE) or were vaccinated by i.m. injection on day 0 with 5 μg HA derived from strain A/Panama/2007/99 (H3N2) formulated as WIV, VS or SU vaccine and were i.n. infected on day 28 with A/Panama virus. Three days later mice were sacrificed. HAI titers and VN titers were determined in individual sera as described in Material & Methods. Results are given as log_2 titers. The detection limit was 2 for HAI determination and 5.3 for VN determination, respectively. Significant ($p<0.05$) and highly significant ($p<0.01$) differences between WIV and the other vaccine formulations are indicated by * and **, respectively.*

The quality of the vaccine-induced immune response was investigated by determination of the IgG subclass profile and enumeration of IFNγ- and IL4-producing T helper cells. As a reference, we included in these studies mice that were immunized by exposure to live virus 28 days prior to virus challenge. Determination of IgG subclasses by ELISA revealed that Balb/c mice which had been earlier exposed to live virus produced similar amounts of IgG1 and IgG2a. Immunization with WIV resulted in the production of substantial amounts of IgG2a but little IgG1 (Fig. 2). Production of antibodies in response to immunization with VS or SU vaccine was lower and the antibodies synthesized were almost exclusively of the IgG1 subclass. C57BL/6 mice express the antibody

subclass IgG2c instead of IgG2a. IgG2c was produced after exposure to live virus and especially after immunization with WIV but was not induced by VS or SU. IgG1 responses to virus and WIV in C57BL/6 mice were similar to those in Balb/c mice while IgG1 responses to VS and SU were lower.

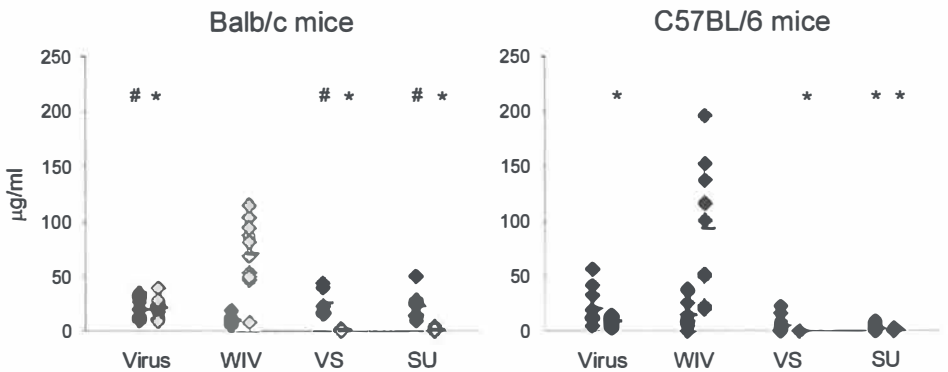

Figure 2: IgG subtypes after immunization and subsequent virus challenge.
*Mice were treated as described in the legend to Fig. 1. An additional group of mice was i.n. infected on day 0 with 150 HAU of live A/Panama virus (virus) and received a second dose of virus 150 HAU on day 28. IgG1 (black diamonds) and IgG2a (light grey diamonds) or IgG2c (dark grey diamonds) were determined by ELISA and amounts were calculated using IgG1, IgG2a, and IgG2c standards. Responses significantly lower or higher than those induced by WIV ($p<0.05$) are indicated by # and *, respectively.*

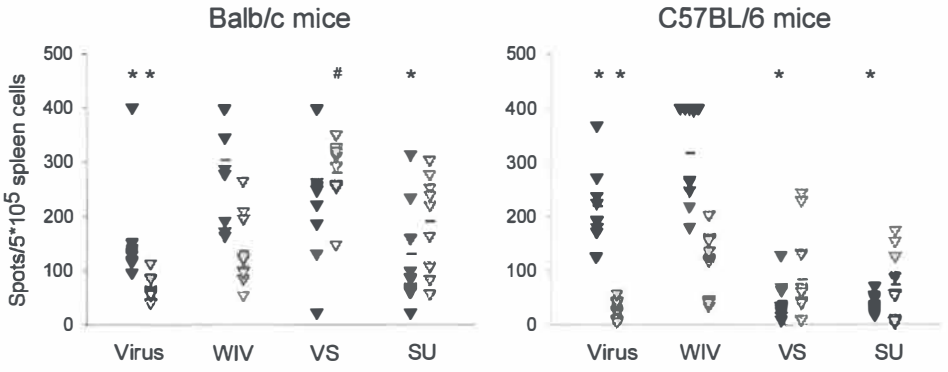

Figure 3: T helper responses after immunization followed by virus challenge.
*Splenocytes obtained from the mice described above were used to perform ELISPOT assays for enumeration of IFNγ- (black triangels) and IL4- (grey triangels) producing T helper cells. Cells were stimulated overnight before lysis and detection of the respective cytokines. Responses significantly lower or higher than those induced by WIV ($p<0.05$) are indicated by # and *, respectively.*

To obtain further insight into the quality of the immune response induced by the three different vaccines in relation to virus exposure, T helper responses were measured (Fig. 3). After earlier virus exposure or immunization with WIV, Balb/c mice as well as C57BL/6 mice generated large numbers of IFNγ-producing cells after challenge. IL4-producing cells were also detected although in much lower amounts. In contrast, the T helper responses to VS and SU vaccine were either balanced or dominated by IL4-producing cells. The number of IFNγ-producing cells in VS- or SU-immunized mice was significantly lower than in WIV-immunized mice except for VS-immunized Balb/c mice ($p<0.01$ in all cases). IFNγ and IL4 are regarded as signature cytokines of Th1 and Th2 cells, respectively. We therefore used the results of the ELISPOT assays to calculate ratios of Th1-type cytokine and Th2-type cytokine producing cells (Th1/Th2 ratios). Ratios were calculated for individual mice and the average and standard deviation were determined per experimental group (Fig. 4). As was found for virus-exposed mice the mean Th1/Th2 ratio was >>1 for all mice immunized with WIV with mean ratios of 2.68 for Balb/c mice and 3.51 for C57BL/6 mice. In contrast, Th1/Th2 ratios in VS- and SU-immunized mice were close to 1 or lower than 1 and in each of these experimental groups a minority of the mice showed ratios >1. Taken together the results of the immunization experiments indicate that immunization with WIV induces a Th1 response with induction of IgG2a and IFNγ-producing T helper cells. At least with respect to the T helper cells this response resembles that observed after exposure to live virus. In contrast, VS and SU vaccines induce Th2 responses dominated by IgG1 and IL4.

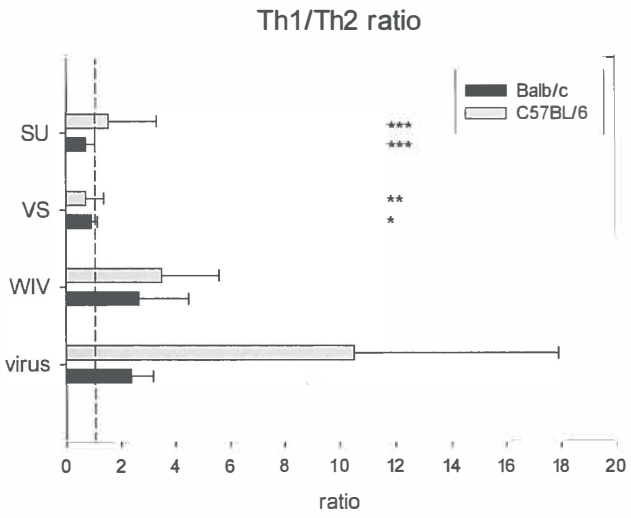

Figure 4: Ratios of IFNγ-producing and IL4-producing T cells (Th1/Th2 ratio) after immunization and challenge.*Ratios were calculated for each individual mouse and are given as mean (+/- standard deviation) per experimental group. A ratio of 1 representing a perfectly balanced response is indicated by a stipple line. *** 3/9 mice ratio >1, **2/10 mice ratio >1, *1/10 mice ratio >1.*

Since dendritic cells (DCs) are important for the polarization of Th cells into Th1 or Th2, the effect of the three vaccine formulations on conventional DCs (cDCs) and plasmacytoid DCs (pDCs) was evaluated *in vitro*. cDCs were generated from Balb/c bone marrow cells by culture in the presence of GM-CSF for 10 days. When exposed to live virus or WIV these cells produced substantial amounts of the proinflammatory cytokines IL1β, IL6 and TNFα (Fig. 5). Exposure to VS also induced these cytokines although to lower extents while exposure to SU had little or no effect on cytokine secretion. Active virus and WIV also induced the secretion of IL12, being known as a key inducer of cellular immune responses. In contrast to the proinflammatory cytokines which were present early after exposure secretion of IL12 was retarded. Similarly, secretion of IL10, mainly involved in control of the immune response, was found at later time points after start of the exposure. IFNγ, which is an important mediator of cellular immune responses and stimulates the production of IgG2a was not secreted after exposure to either of the vaccines. This result is in line with an earlier study which reports on in vitro production of IFNγ by bone marrow DCs cultured in GM-CSF + IL-15 but not DCs cultured in GM-CSF only as used here [35].

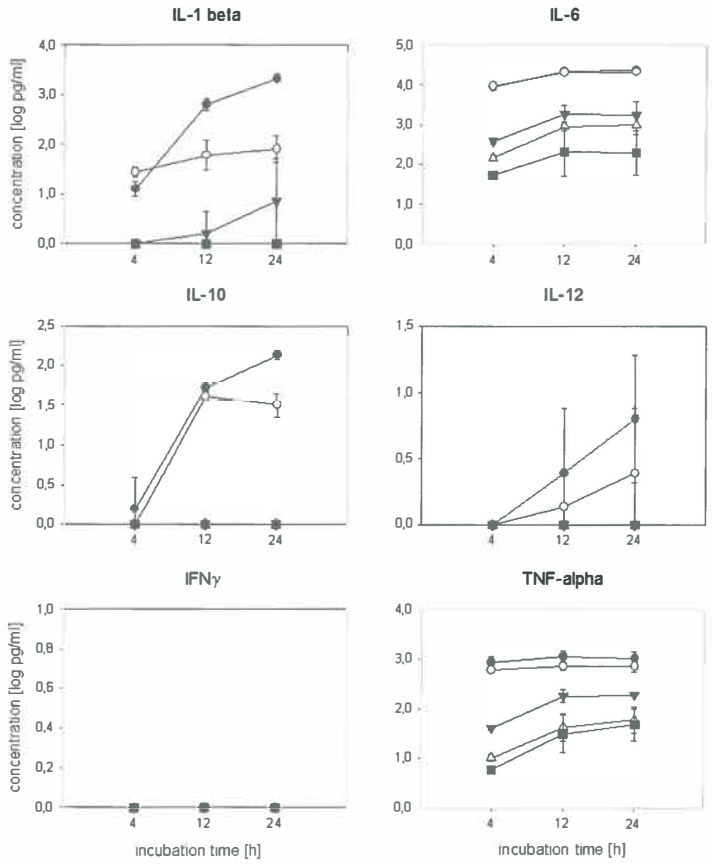

Figure 5: Cytokine production of conventional DCs upon exposure to virus or vaccines. *Bone marrow cells were cultured for 9 days with GM-CSF to obtain cDCs. On day 9 cells were exposed to live influenza virus (closed circles), WIV (open circles), VS (closed triangles) or SU (open triangles) for the periods indicated or were left untreated (black squares). Supernatants were harvested and cytokines were determined using Luminex technology. Results shown are the mean of two independent experiments. Each supernatant was measured in duplicate.*

pDCs have been described as the major producers of type I interferon during virus infection [36]. A pDC-enriched cell population was prepared from splenocytes of naïve Balb/c mice and the cells were incubated with 0.01, 0.1, 1, or 10 μg HA formulated as active virus or WIV, VS or SU vaccine, respectively (Fig. 6a). Cells incubated with active virus or WIV produced substantial amounts of IFNα. These amounts peaked at a HA concentration of 0.1 μg/ml but were lower for lower as well as higher antigen doses. In contrast, pDCs incubated with VS or SU vaccine did not produce detectable amounts of IFNα at any of the antigen concentrations

used. IFNα production in reaction to WIV was also observed in crude splenocyte preparations but amounts increased significantly upon pDC enrichment indicating pDCs as a major source of IFNα (Fig. 6b). Similar to spleen-derived pDCs from Balb/c mice, pDCs derived from bone marrow of C57BL/6 mice by culture with Flt3 ligand produced IFNα upon exposure to WIV but not to SU vaccine (Fig. 6c). These data indicate that the differential reaction to the different vaccine formulations is independent on the source of the pDCs and the strain of mice. In conclusion, WIV is superior to VS and SU in activating cDCs as well as pDCs to produce cytokines that can modulate the extent and the phenotype of adaptive immune responses.

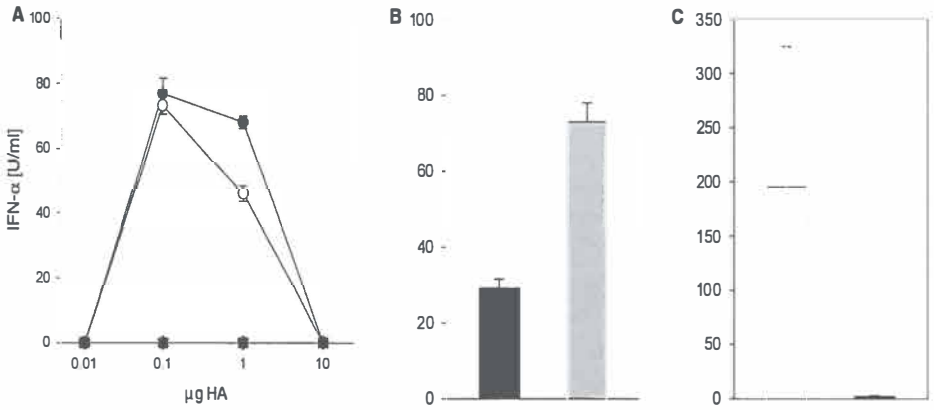

Figure 6: Production of IFNα by plasmacytoid DCs after exposure to virus or vaccines.
(A) Splenocytes derived from Balb/c mice were enriched for pDCs as described in Material & Methods. Cells were incubated for 20 hours with the indicated amounts of HA using either live virus (filled circles), WIV (open circles), VS (filled triangles), or SU vaccine (open triangles). Supernatants were harvested and IFNα was determined by sandwich ELISA. Results of a representative experiment are shown. (B) Crude splenocytes (black bar) and splenocytes enriched for pDCs as above (grey bar) were incubated for 20 hours with 0.1 µg WIV. Supernatants were analyzed for IFNα as above. (C) Flt3 ligand cultured bone marrow cells were exposed to 0.1 µg WIV (white bar) or SU vaccine (black bar for 20 hours and IFNα in the supernatants was analyzed as above.

Discussion

In this paper we show that, using the same amount of HA, immunization of mice with WIV results in higher HAI and VN titers than immunization with VS or SU. Moreover, the quality of the response to the vaccines differs profoundly. While WIV elicits a Th1 response reminiscent of that found after virus infection, VS and SU induce typical Th2 responses. The observed differences in the amounts and subtypes of the induced antibodies and the phenotypes of the T cell responses to the different vaccine formulations were independent of antigen dose as revealed by dose-response studies using antigen amounts as low as 0.04 μg (results not shown). The responses found *in vivo* reflected the effects of the vaccines on dendritic cells *in vitro*. Similar to active virus WIV induced the synthesis of various cytokines by cDCs and stimulated the production of type I interferon by pDCs. In contrast, VS and SU had only moderate to low effects on DCs *in vitro*.

Similar to the results obtained in the murine system reported here, higher HAI titers in response to WIV as compared to the other formulations have also been reported from human clinical trials especially when the study population was naive to the vaccine strain used [7,37-39]. The superior immunogenicity of WIV could be of great importance in a pandemic situation when protective immune responses against a new virus variant have to be achieved with a minimum amount of antigen.

Evidence is accumulating that not only the magnitude of the immune response but also its quality is important for protection. In the murine system IgG2a was found recently to have a greater protective potential than IgG1 [22, Bungener et al, unpublished observations]. Moreover, Th1 cells can protect from lung damage while Th2 cells can be deleterious [24, 26-28]. These observations imply that the vaccine-induced immune response should ideally be of a type I phenotype. In the murine system, the current study and studies of others show that only WIV but not split, VS, or SU vaccine can induce the desired type I response [40-43]. In the human situation the quality of the evoked immune response has so far largely been neglected. HAI titers, and for H5N1 trials VN titers, are usually the only correlates of protection measured in clinical studies. Since vaccines can differ profoundly in the type of response they evoke and since this type can have important implications for protection we strongly recommend including the determination of immune response quality in future clinical studies.

Interestingly, the differences we found in the immune response to active virus and WIV on the one hand and VS and SU on the other hand correlated with the effect of these agents on dendritic cells *in vitro*. DCs are considered as the most important antigen presenting cells and are the only cells that can activate naive T cells [for reviews see 44-46]. Moreover, they are involved in the polarization of naive T cells to a Th1 or Th2 phenotype, respectively, and do so by the secretion of defined cytokines [for review see 47]. We used bone marrow cells cultured with GM-CSF to generate cDCs *in vitro*. Only when incubated with active virus or WIV these cells produced substantial amounts of cytokines. These included IL12 which is considered as necessary to activate IFNγ production by Th1 cells [35]. Virus and WIV also induced the production of the proinflammatory cytokines IL1β, IL6 and TNFα. These cytokines were also found to be upregulated in human DCs incubated with live influenza virus [48]. Notably, IL10 which is involved in the control of the immune response and considered as a Th2 cytokine was also induced by virus and WIV although at later time points as the proinflammatory cytokines. cDC-derived cytokines are very important for activating, directing and controlling adaptive immune responses. The higher and qualitatively different immune responses evoked by WIV as compared to the other vaccines might therefore – at least partly – be explained by stronger effects of WIV on cDCs.

pDCs are less active in antigen presentation than cDCs. However, they are highly important in the innate defense of virus infections since they can produce large amounts of type I interferons, particularly IFNα upon exposure to virus [36,49,50]. In our assays, pDCs exposed to active virus and WIV produced similar amounts of IFNα whereas neither VS nor SU induced any IFNα synthesis. Type I interferons are major inducers of activation of immature DCs and lead to the upregulation of MHC molecules, chemokines, chemokine receptors and co-stimulatory molecules [50]. Moreover, they exert direct effects on B and T cells thus affecting antibody secretion and antibody class switching [51]. Type I IFNs have been described as natural adjuvants. When added during immunization with influenza vaccine, type I IFN enhanced IgG1 but especially IgG2a responses and significantly improved survival of the mice after virus challenge [52]. We therefore consider it likely that IFNα presumably produced by pDCs, upon immunization with WIV but not VS or SU vaccine is responsible for the enhanced immune response and the dominant Th1 reaction.

Together with studies of others comparing split vaccine with WIV [41-43] our current investigations allow interesting conclusions on the parameters which

determine vaccine immunogenicity. Split vaccine and WIV both contain all the viral components yet they elicit very different immune responses indicating that not only vaccine composition but also the physical structure of the vaccine (soluble vs particulate) is important. On the other hand, virosomes and WIV sharing the particulate structure but differing in their composition also induce very different responses. From these results we conclude that it is the combination of vaccine components present and their physical organization which determines the immunological properties of a vaccine. Interestingly, WIV and active virus elicited quantitatively and qualitatively similar responses indicating that the structural integrity of the virus particles is more important for the immune response than the presence or absence of virus replication. The exact mechanisms by which active virus and the vaccines induce the differential production of DC cytokines are so far unclear. It is tempting to speculate that stimulation of pathogen associated molecular pattern (PAMP) receptors is involved. Elucidation of these mechanisms is highly interesting in the context of rational vaccine development and will be approached in a follow-up study.

In conclusion, we showed that WIV vaccine induces a stronger and more Th1-skewed immune response than VS and SU vaccines most probably due to direct action of the vaccine on conventional as well as plasmacytoid DCs. Due to its enhanced immunogenicity WIV can induce protective immune responses at lower antigen doses. Moreover, the type of the immune response elicited by WIV has proven to provide better protection in animal models. We therefore consider WIV as a highly attractive vaccine candidate especially in a pandemic situation.

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References

1. Stöhr K, Kieny M, Wood D. Influenza pandemic vaccines: how to ensure a low-cost, low-dose option. Nat Rev Micro 2006; 4: 565-566.
2. World Health Organization. Avian influenza assessing the pandemic threat. (www.who.int/crs/desease/infuenza/WHO CDS 2005 29/en/) 2005.
3. Murray CJ, Lopez AD, Chin B, Feehan D, Hill KH. Estimation of potential global influenza mortality on the basis of vital registry data from the 1918-20 pandemic: a quantitative analysis. The Lancet 2006; 368: 2211-18.
4. Wilschut JC, McElhaney JE, Palache AM. Vaccination: cornerstone of influenza control. In: Wilschut JC, McElhaney JE, Palache AM, editors. Influenza. Elsevier, 2006: 150-183.
5. Stegmann T, Morselt HW, Booy FP, van Breemen JF, Scherphof G, Wilschut J. Functional reconstitution of influenza virus envelopes. EMBO J 1987 6, 2651–59.
6. Huckriede A, Bungener L, Daemen T, Wilschut J. Influenza virosomes in vaccine development. Meth Enzymol 2003; 373: 74-91.
7. Stephenson I, Nicholson KG, Glück R, Mischler R, Newman RW, Palache AM et al. Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. The Lancet 2003; 362: 1959-66.
8. Hehme N, Engelmann H, Kuenzel W, Neumeier E, Saenger R. Immunogenicity of a monovalent, aluminum-adjuvanted influenza whole virus vaccine for pandemic use. Virus Research 2004; 103: 163-171.
9. Hehme N, Engelmann H, Kunzel W, Neumeier E, Sanger R. Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. Med Microbiol Immunol (Berl) 2002; 191: 203-208.
10. Bresson J, Perronne C, Launay O, Gerdil C, Saville M, Wood J et al. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. The Lancet 2006; 367: 1657-1664.
11. Lin J, Zhang J, Dong X, Fang H, Chen J, Su N et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. The Lancet 2006; 368: 991-997.
12. de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaus AD. Haemagglutination-inhibiting antibody to influenza virus. Dev Biol (Basel) 2003; 115: 63-73.
13. Coutelier JP, van der Logt JT, Heessen FW, Warnier G, Van Snick J. IgG2a restriction of murine antibodies elicited by viral infections. J Exp Med 1987; 165: 64-69.
14. Hocart MJ, Mackenzie JS, Stewart GA. The immunoglobulin G subclass response of mice to influenza A virus: the effect of mouse strain, and the neutralizing abilities of individual protein A-purified subclass antibodies. J GenVirol 1998; 70: 2439-48.

15. Fazekas G, Rosenwirth B, Dukor P, Gergely J, Rajnavolgyi E. IgG isotype distribution of local and systemic immune responses induced by influenza virus infection. Eur J Immunol 1994; 24: 3063-3067.

16. Huber VC, Lynch JM, Bucher DJ, Le J, Metzger DW. Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. J Immunol 2001; 166: 7381-7388.

17. Neuberger MS, Rajewsky K. Activation of mouse complement by monoclonal mouse antibodies. Eur J Immunol 1981; 11:1012-1016.

18. Heusser CH, Anderson CL, Grey HM. Receptors for IgG: subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line. J Exp Med 1977; 145: 1316-1327.

19. Gessner JE, Heiken H, Tamm A, Schmidt RE. The IgG Fc receptor family. Ann Hematol 1998; 76: 231-248.

20. Mozdzanowska K, Feng J, Eid M, Zharikova D, Gerhard W. Enhancement of neutralizing activity of influenza virus-specific antibodies by serum components. Virology 2006; 352: 418-426.

21. Jayasekera JP, Moseman EA, Carroll MC. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. J Virol 2007; 81: 3487-3494.

22. Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA et al. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol 2006; 13: 981-990.

23. Brown D, Roman E, Swain S. CD4 T cell responses to influenza infection. Seminars in Immunology 2004; 16: 171-177.

24. Moran TM, Isobe H, Fernandez-Sesma A, Schulman JL. Interleukin-4 causes delayed virus clearance in influenza virus-infected mice. J Virol 1996; 70: 5230-5235.

25. Moran TM, Park H, Fernandez-Sesma A, Schulman JL. Th2 responses to inactivated influenza virus can be converted to Th1 responses and facilitate recovery from heterosubtypic virus infection. J Infect Dis 1999; 180: 579-585.

26. Bot A, Bot S, Bona C. Protective Role of Gamma Interferon during the Recall Response to Influenza Virus. J Virol 1998; 72: 6637-6645.

27. Bot A, Holz A, Christen U, Wolfe T, Temann A, Flavell R et al. Local IL-4 Expression in the lung reduces pulmonary influenza-virus-specific secondary cytotoxic T cell responses. Virology 2000; 269: 66-77.

28. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. Emerg Infect Dis 2006; 12: 48-54.

29. Swain S, Agrewala J, Brown D, Jelley-Gibbs D, Golech S, Huston G et al. CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. Immunol Rev 2006; 211: 8-22.

30. Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol. 1999; 37: 937-43.

31. Bungener L, Huckriede A, de Mare A, de Vries-Idema J, Wilschut J, Daemen T, Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity, Vaccine 2005; 23: 1232–41.

32. Lutz M, Kukutsch N, Ogilvie A, Rossner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 1999; 223: 77-92.

33. Vignali DA. Multiplexed particle-based flow cytometric assays. J Immunol Methods 2000; 243: 243–55.

34. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A. 2004;101: 5598-603.

35. Feili-Hariri M, Falkner D, Morel P. Polarization of naive T cells into Th1 or Th2 by distinct cytokine-driven murine dendritic cell populations: implications for immunotherapy. J Leukocyte Biol 2005; 78: 656-664.

36. Barchet W, Blasius A, Cella M, Colonna M. Plasmacytoid dendritic cells: in search of their niche in immune responses. Immunol Res 2005; 32:75-83.

37. Kark JD, Lebiush M, Rannon L, Witztum E, Nili E, Kedem R. The antigenicity of whole virus versus subunit trivalent influenza vaccines -- a field trial in the Israel Defence Forces, 1978. Med Microbiol Immunol 1981; 170: 55-62.

38. Balkovic ES, Florack JA, Six HR. Immunoglobulin G subclass antibody responses of mice to influenza virus antigens given in different forms. Antiviral Res 1987; 8:151-160.

39. Bernstein DI, Zahradnik JM, DeAngelis CJ, Cherry JD. Clinical reactions and serologic responses after vaccination with whole-virus or split-virus influenza vaccines in children aged 6 to 36 months. Pediatrics 1982; 69: 404-408.

40. McElhaney JE, Meneilly GS, Lechelt KE, Beattie BL, Bleackley RC. Antibody response to whole-virus and split-virus influenza vaccines in successful ageing. Vaccine 1993; 11:1055-1060.

41. Hovden AO, Cox RJ, Haaheim LR. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol 2005; 62: 36-44.

42. Hovden AO, Cox RJ, Madhun A, Haaheim LR. Two doses of parenterally administered split influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral shedding upon challenge in mice. Scand J Immunol 2005; 62: 342-352.

43. Szyszko E, Brokstad K, Cox RJ, Hovden AO, Madhun A, Haaheim LR. Impact of influenza vaccine formulation with a detailed analysis of the cytokine response. Scand J Immunol 2006; 64: 467-475.

44. Guermonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 2002; 20: 621-667.

45. Shortman K, Liu Y-J. Mouse and human dendritic cell subtypes. Nat Rev Immunol 2002; 2: 151-61.

46. Lopéz C, Yount J, Moran T. Toll-Like Receptor-Independent Triggering of Dendritic Cell Maturation by Viruses. J Virol 2006; 80:3128-3134.

47. Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. Nat Immunol 2000; 1:199-205.

48. Huang Q, Liu D, Majewski P, Schulte LC, Korn JM, Young RA et al. The plasticity of dendritic cell responses to pathogens and their components. Science 2001; 294: 870-875.

49. Asselin-Paturel C, Boonstra A, Dalod M, Durand I, Yessaad N, Dezutter-Dambuyant C et al. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat Immunol 2001; 2:1144-1150.

50. Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons (alpha/beta) in immunity and autoimmunity. Annu Rev Immunol 2005; 23: 307-36.

51. Le Bon A, Thompson C, Kamphuis E, Durand V, Rossmann C, Kalinke U et al. Cutting edge: Enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. J Immunol 2006; 176: 2074-2078.

52. Proietti E, Bracci L, Puzelli S, Di Pucchio T, Sestili P, De Vincenzi E et al. Type I IFN as a natural adjuvant for a protective immune response: Lessons from the influenza vaccine model. J Immunol 2002; 169: 375-383.

Superior Immunogenicity of Inactivated Whole Virus H5N1 Influenza Vaccine is Primarily Controlled by Toll-like Receptor Signalling

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Abstract

In the case of an influenza pandemic, the current global influenza vaccine production capacity will be unable to meet the demand for billions of vaccine doses. The ongoing threat of an H5N1 pandemic therefore urges the development of highly immunogenic, dose-sparing vaccine formulations. In unprimed individuals, inactivated whole virus (WIV) vaccines are more immunogenic and induce protective antibody responses at a lower antigen dose than other formulations like split virus (SV) or subunit (SU) vaccines. The reason for this discrepancy in immunogenicity is a long-standing enigma. Here, we show that stimulation of Toll-like receptors (TLRs) of the innate immune system, in particular stimulation of TLR7, by H5N1 WIV vaccine is the prime determinant of the greater magnitude and Th1 polarization of the WIV-induced immune response, as compared to SV- or SU-induced responses. This TLR dependency largely explains the relative loss of immunogenicity in SV and SU vaccines. The natural pathogen-associated molecular pattern (PAMP) recognized by TLR7 is viral genomic ssRNA. Processing of whole virus particles into SV or SU vaccines destroys the integrity of the viral particle and leaves the viral RNA prone to degradation or involves its active removal. Our results show for a classic vaccine that the acquired immune response evoked by vaccination can be enhanced and steered by the innate immune system, which is triggered by interaction of an intrinsic vaccine component with a pattern recognition receptor (PRR). The insights presented here may be used to further improve the immune-stimulatory and dose-sparing properties of classic influenza vaccine formulations such as WIV, and will facilitate the development of new, even more powerful vaccines to face the next influenza pandemic.

Author Summary

The rise and spread of the highly pathogenic avian H5N1 influenza virus has seriously increased the risk of a new influenza pandemic. However, the number of vaccine doses that can be produced with today's production capacity will fall short of the demand in times of a pandemic. Use of inactivated whole virus (WIV) vaccines, which are more immunogenic than split virus or subunit vaccines in an unprimed population, could contribute to a dose-sparing strategy. Yet, the mechanisms underlying the superior immunogenicity of WIV vaccine formulations are unknown. Here, we demonstrate that the viral RNA present in inactivated virus particles is crucial for the improved immunogenic properties of WIV in mice. By triggering Toll-like receptor 7 (TLR7), the viral RNA activates innate immune mechanisms that augment and determine subsequent adaptive responses. Efficient TLR7 signalling is lost in split virus and subunit vaccines with the processing steps that lead to disruption of the integrity of the virus particle and exclusion of the RNA. Our results prove for the first time to our knowledge that the immune-potentiating mechanism of a classic vaccine is based on activation of the innate immune system by one of its structural components. These findings may reflect a general principle for viral vaccines and provide a rational basis for further improvement of influenza vaccines, which are urgently needed in the face of the current H5N1 pandemic threat.

Introduction

The first cases of human infection with highly pathogenic avian influenza (HPAI) H5N1 virus occurred in 1997 during an outbreak in Hong Kong [1]. Since then HPAI H5N1 has spread across Asia, Europe, Africa and the Pacific, and has caused a cumulative number of 338 laboratory confirmed human cases of infection, with a fatality rate of >60% [2]. Although no sustained human to human transmission has been observed yet, the threat of an imminent H5N1 pandemic requires maximum preparedness [3]. Vaccination is considered the cornerstone of protection against epidemic and pandemic influenza. However, an anticipated scarcity of the antigenic vaccine components and a narrowed time window between vaccine production and deployment puts special constraints on the vaccine formulation to be used in a pandemic situation [4],[5]. Consequently, pandemic vaccine formulations should ideally be dose sparing and uncomplicated to produce [6],[7].

Whole inactivated virus (WIV) vaccines consisting of formalin-inactivated whole virus particles were the first registered influenza vaccines licensed in 1945 in the United States [8]. However, the use of this vaccine formulation caused a relatively high incidence of adverse events, including local reactions at the site of injection and febrile illness, particularly among children [9],[10]. In the 1960 and 1970s, WIV vaccines were therefore largely replaced by less reactogenic split virus (SV) and subunit (SU) formulations [8]. SV and SU vaccines contain detergent- and/or ether-disrupted (split) virus particles or purified viral haemagglutinin (HA) and neuraminidase (NA) proteins, respectively. Apparently, disruption of whole inactivated influenza virus particles diminishes the reactogenicity of the vaccines.

In primed individuals, unadjuvanted WIV, SV, and SU vaccines in general induce similar immune responses in terms of haemagglutination inhibition (HI) titres (for a meta-analysis over 24 studies see[11]). However, in individuals that have not been exposed to the vaccine antigens before, WIV vaccines are more immunogenic than SV and SU vaccines [9],[11],[12]. Similarly, in naïve animals immunization with WIV raises stronger immune responses than immunization with SV or SU [13]–[15], especially after a single administration. In the case of an H5N1 pandemic, the majority of the population is expected to be immunologically naïve to the H5N1 subtype. In this scenario, use of WIV as basis for an optimized vaccine may be of advantage, for its immunogenic superiority seems to rely on the

ability to activate unique mechanisms in the priming event of the immune response.

Thus, WIV seems to harbour an intrinsic immune-potentiating component that is lost during processing of inactivated virus particles to SV and SU vaccine formulations. In earlier experiments, we and others observed that immunization of mice with WIV vaccine results in a Th1-skewed immune response and strong antibody induction with high levels of IgG2a antibodies [14]–[16]. This response type was found irrespective of the murine genetic background or subtype of virus (either H1N1 or H3N2) and conferred protective immunity against challenge with homologous virus [15],[16]. By contrast, immunization with SU vaccine yielded responses of a Th2 phenotype with lower antibody levels mainly consisting of the IgG1 subtype, which did not lead to protection. "Empty" reconstituted viral envelopes (virosomes) resembling intact virus particles but devoid of the viral nucleocapsid elicited responses similar to those after vaccination with SU formulations [15]. This identifies the viral nucleocapsid which contains the viral genomic ssRNA as the immune-potentiating component of WIV.

In the past decade, it has become increasingly clear that the acquired immune response to microbial infection is regulated through recognition of pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) and other pattern recognition receptors of the innate immune system [17]–[20]. However, the importance of TLR signalling in immune responses to vaccines remains largely unclear. A recent study showed that TLR signalling is not important for the antibody-enhancing effect of classical vaccine adjuvants such as Complete Freund's adjuvant (CFA) [21]. Since CFA contains dried mycobacteria, and therefore mycobacterial PAMPs [17], this observation casts doubt on the importance of PAMPs and TLRs in augmenting immune responses to vaccination. Influenza viral genomic ssRNA is a natural PAMP recognized by TLR7 [22]. Here, we investigate whether PAMP recognition by TLRs, in particular recognition of viral ssRNA by TLR7, is responsible for the superior response to WIV vaccines compared to SV and SU influenza vaccine formulations.

Results/Discussion

To analyze the role of ssRNA and other PAMPs in the response to influenza vaccines in detail, we immunized wild-type C57BL/6 mice, TLR7 knock-out mice, and MyD88/TRIF double knock-out mice with different vaccine formulations. MyD88 (myeloid differentiation factor 88) is an adaptor molecule which functions

downstream of all known TLRs and IL1R family members with the exception of TLR3, which instead recruits a MyD88-related adapter molecule, TRIF (TIR domain-containing adaptor protein inducing interferon β) [17]. Consequently, a deficiency of both MyD88 and TRIF excludes signalling by all TLRs. Mice were immunized intramuscularly with β-propiolactone-inactivated H5N1 (NIBRG-14) WIV, SV, or SU vaccine. Quantitative PCR using primers specific for segment 7 of the viral genome revealed that WIV contained per vaccine dose at least 5×10^8 copies of viral RNA, the natural ligand of TLR7. In SU or SV vaccine the amount of RNA was 500 and 5,000 times lower than in WIV, respectively. Four weeks after immunization, serum and spleen cells were collected for evaluation of humoral and cellular immune responses.

Serum HI titres in WIV-immunized $TLR7^{-/-}$ mice and $MyD88^{-/-}/TRIF^{-/-}$ mice were found to be significantly lower than in WIV-immunized wild-type mice (Figure 1A; $p = 0.021$ and $p = 0.001$, respectively). Although sera from $TLR7^{-/-}$ mice immunized with WIV showed a higher geometric mean titre (GMT) than sera from WIV-immunized $MyD88^{-/-}/TRIF^{-/-}$ mice, this difference was not significant ($p = 0.053$). Most of the HI titres of SV- and SU-immunized wild-type mice were below detection level, precluding evaluation of the effect of the knock-out mutations on the HI responses to these vaccines.

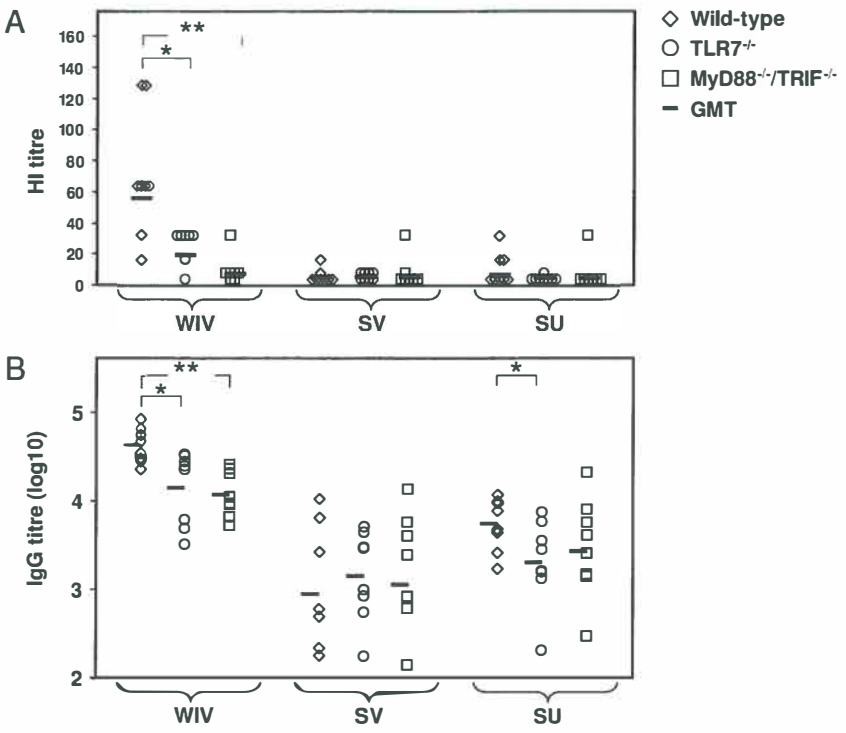


Figure 1. TLRs contribute to the efficacy of H5N1 WIV vaccine. *Four weeks after immunization of wild-type, TLR7–/–, and MyD88–/–/TRIF–/– mice with WIV, SV, or SU vaccine (5 μg HA), serum HI titres (A) and H5N1-specific IgG titres (B) were determined for the individual mice. Titres below the detection limit were assigned with half the value of the lowest detectable serum dilution, which was 8 in the HI assay and 100 in the IgG ELISA. Significant ($p<0.05$) and highly significant ($p<0.01$) differences between wild-type mice and mutant mice receiving the same vaccine are indicated by * and **, respectively. GMT indicates geometric mean titter.*

Similar to the HI titres, virus neutralization (VN) titres of pooled serum samples from mice immunized with WIV were lower in the knock-out groups than in the wild-type group (Table 1). These results clearly show that TLR signalling is critically involved in the response to WIV immunization. Yet, in the knock-out groups, VN titres obtained after immunization with WIV were still modestly higher than those obtained after vaccination of wild-type mice with the other vaccines. This points to TLR-independent pathways contributing to the superior antibody response to WIV vaccine.

Table 1. TLR-dependent and -independent mechanisms contribute to virus neutralization titres induced by WIV.

Vaccine	Mouse Strain	VN Titre
	wt	640
WIV	TLR7$^{-/-}$	80
	MyD88$^{-/-}$/TRIF$^{-/-}$	80
	wt	20
SV	TLR7$^{-/-}$	20
	MyD88$^{-/-}$/TRIF$^{-/-}$	40
	wt	40
SU	TLR7$^{-/-}$	20
	MyD88$^{-/-}$/TRIF$^{-/-}$	20

Mouse sera were collected 4 wk after immunization with different vaccine formulations and pooled per immunization group (n = 8 per group, except for SU immunized MyD88$^{-/-}$/TRIF$^{-/-}$ mice: n = 7) and subsequently submitted to the VN assay.

Serum titres of H5N1-specific IgG were determined by ELISA. In accordance with the HI and VN results, IgG titres were significantly decreased in WIV-immunized TLR7$^{-/-}$ and MyD88$^{-/-}$/TRIF$^{-/-}$ mice compared to wild-type mice (Figure 1B; p = 0.010 and p = 0.001, respectively). However, like the VN titres, the IgG titres in the WIV-immunized mutant mice were still significantly higher than those induced by SV (TLR7$^{-/-}$: p = 0.001; MyD88$^{-/-}$/TRIF$^{-/-}$: p = 0.005) or SU (TLR7$^{-/-}$: p = 0.005; MyD88$^{-/-}$/TRIF$^{-/-}$: p = 0.021) immunization again indicating involvement of TLR-independent pathways. The relative contributions of TLR-dependent and -independent mechanisms to the superior IgG response to WIV can be estimated by comparing the difference in geometric mean titre (GMT) between WIV-immunized wild-type and MyD88/TRIF-deficient mice with the difference between WIV-immunized wild-type mice and SV- or SU-immunized wild-type mice. Using this procedure the TLR-dependent contribution was calculated to be 73% and 83% for WIV versus SV and WIV vs SU, respectively (for calculation, see Text S1). The IgG responses to SV and SU vaccine in both TLR7$^{-/-}$ or MyD88$^{-/-}$/TRIF$^{-/-}$ mice did not differ from those in wild-type mice, except for the IgG response to SU in TLR7$^{-/-}$ mice, which was slightly but significantly decreased (p = 0.038; Figure 1B). Together with the HI and VN results, these findings demonstrate that the superior antibody response to WIV is

predominantly regulated by TLRs, TLR7 in particular, while TLRs do not seem to play a prominent role in SV and SU antibody responses.

We next investigated the role of TLRs in the Th1 polarization of the response characteristically found after WIV vaccination. We first assessed numbers of IFNγ- and IL4- producing T cells (Th1 and Th2 cells, respectively) in a cytokine-specific Elispot assay, after re-stimulation of spleen cells from immunized mice with H5N1 SU vaccine. Numbers of Th1 cells were significantly decreased in WIV-immunized knock-out mice compared to wild-type mice ($p = 0.003$ and $p = 0.010$ for TLR7$^{-/-}$ and MyD88$^{-/-}$/TRIF$^{-/-}$ mice, respectively), and matched those found in SV- and SU-immunized wild-type mice (Figure 2). No difference was found between TLR7$^{-/-}$ and MyD88$^{-/-}$/TRIF$^{-/-}$ mice. Numbers of influenza-specific IL4-producing cells were extremely low in all animals for all vaccine formulations without significant differences between knock-out and wild-type mice (not shown). These data indicate that stimulation of TLR7 by ssRNA is the predominant determinant of the strong Th1-type cellular response induced by WIV.

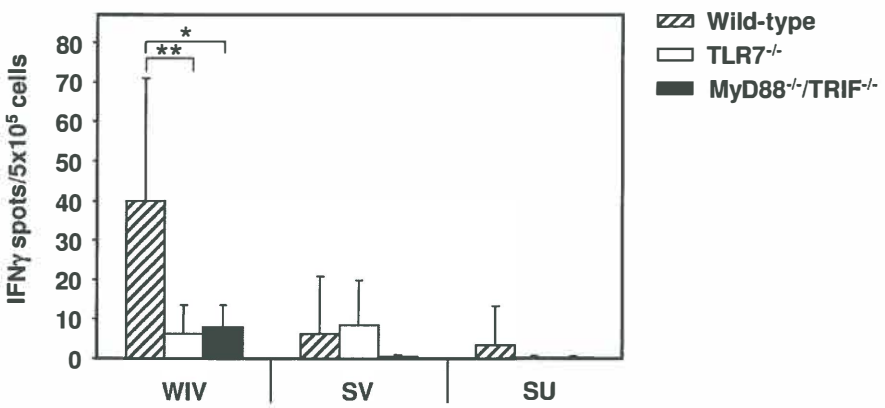


Figure 2. Induction of IFNγ-producing T cells by H5N1 WIV vaccine depends on TLR7 signalling. *Spleen cells of wild-type mice and mutant mice immunized with WIV, SV, or SU vaccine were re-stimulated in vitro with SU vaccine, and numbers of IFNγ-producing cells were determined by Elispot assay. Bars represent the average values of triplicate determinations per mouse for each mouse type and immunization group (n = 8; MyD88–/–/TRIF–/–/SU, n = 7), with standard deviation. Significant (p<0.05) and highly significant (p<0.01) differences between wild-type mice and mutant mice receiving the same vaccine are indicated by * and **, respectively.*

We further determined the subtype profiles of H5N1-specific serum IgG by ELISA (Figure 3). IFNγ is known to stimulate production of IgG2a subtype antibodies by activated B cells, while IL4 stimulates IgG1 secretion [23]. In C57BL/6 mice, however, the IgG2c subtype is produced instead of IgG2a[24],[25]. Hence, a

predominance of IgG2c or IgG1 is indicative of a Th1- or Th2-type response, respectively. WIV immunization of TLR7$^{-/-}$ mice as well as MyD88$^{-/-}$/TRIF$^{-/-}$ mice resulted in significantly reduced IgG2c levels as compared to wild-type mice (Figure 3; $p = 0.001$ for both types of knock-out mice), supporting a role for TLR7 in Th1 polarization. IgG1 was increased in WIV-immunized TLR7$^{-/-}$ mice ($P = 0.050$), adding to the preponderance towards a Th2-type response to WIV in these mice. The average of ratios of serum IgG2c and IgG1 concentrations (determined with appropriate IgG subtype protein standards) was 17.82 (SD 8.44) for the wild-type mice immunized with WIV, compared to 0.53 (SD 0.41) for TLR7$^{-/-}$ mice immunized with WIV. SV and SU vaccines induced predominantly IgG1 and low levels of IgG2c, consistent with a Th2-type response (Figure 3). For reasons unknown, SU vaccine induced lower IgG1 titres in both types of knock-out mice compared to the wild-type mice (TLR7$^{-/-}$: $p = 0.050$; MyD88$^{-/-}$/TRIF$^{-/-}$: $p = 0.014$). Whether the presence of some residual RNA in SU vaccine might play a role remains to be shown.

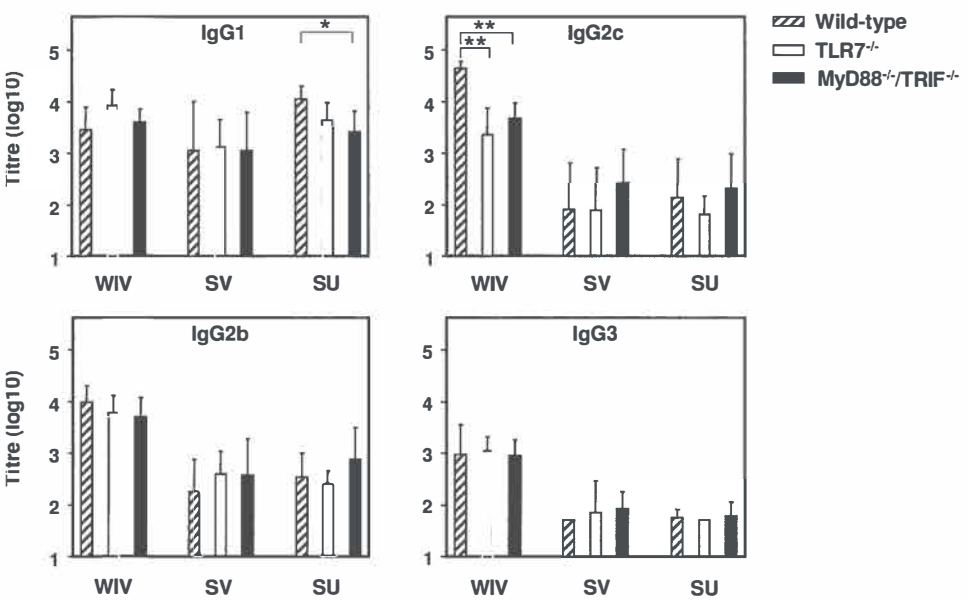


Figure 3. H5N1 WIV vaccine induces Th1-type antibody responses via TLR7 signalling. *Serum titres of H5N1-specific IgG1 subtype (Th2-type antibody), and IgG2c, IgG2b, and IgG3 subtypes (Th1-type antibodies) were determined by ELISA. Geometric mean titres are plotted for each group of wild-type mice or mutant mice (n = 8; MyD88−/−/TRIF−/−/SU, n = 7) immunized with WIV, SV, or SU vaccine. Significant (p<0.05) and highly significant (p<0.01) differences between wild-type mice and mutant mice receiving the same vaccine are indicated by * and **, respectively.*

The response characteristics of the different H5N1 vaccines in wild-type mice were well in line with those previously found for other influenza subtypes [15],[16]. This consistency is supportive of a general mechanism underlying the differences in responses to WIV, SV and SU vaccine, which operates irrespective of the virus subtype used to vaccinate.

The above results demonstrate that TLR signalling plays an important role in the magnitude and Th1 skewing of the response to WIV influenza vaccines. Yet, in TLR-ko mice, WIV remained more immunogenic than SV and SU vaccines, inducing significantly higher titres of total IgG (Figure 1B) and Th1-type antibody subtypes (IgG2b, IgG2c, IgG3; Figure 3; $p<0.05$ for all comparisons). Thus, next to TLR-dependent mechanisms, a (minor) TLR-independent factor seems to contribute to the superior magnitude and Th1-skewing of the immune response to WIV. Type I interferons, including IFNα, have been shown to stimulate antibody responses and isotype switching to IgG2a when added to influenza subunit vaccine or other protein antigens [26],[27], even without the need for additional TLR stimuli. We have previously shown for an H3N2 influenza virus strain that, unlike SU vaccine, WIV vaccine efficiently induced interferon α (IFNα) production in plasmacytoid dendritic cells (pDCs) *in vitro* [15]. We therefore evaluated the induction of IFNα by the H5N1 influenza vaccine formulations used in this study and its TLR7 dependency *in vitro*. In pDCs of wild-type mice cultured from bone marrow cells (Figure 4A, black bars) or enriched from splenocytes (Figure 4B, black bars) WIV but not SV or SU induced IFNα production. In bone marrow-derived pDCs from TLR7$^{-/-}$ mice, IFNα production upon incubation with WIV was strongly decreased as compared to wild-type DCs (Figure 4A), confirming the results of others [22]. However, spleen-derived pDCs from TLR7$^{-/-}$ mice exposed to WIV produced similar amounts of IFNα as compared to pDCs from wt mice (Figure 4B). Thus, while in pDCs cultured from bone marrow induction of IFNα production by WIV is strictly dependent on TLR7, in pDCs enriched directly from spleen cells it is independent of TLR7. This implies that bone marrow pDCs and spleen pDCs are not completely identical. In line with this notion, bone marrow pDCs and spleen pDCs were earlier found to respond differently to HSV virus infection with respect to the TLR9 dependency of the IFNα response [28]. Our results show that WIV is indeed able to induce IFNα in a TLR7-independent way. This may also be the case in the in vivo situation, where in accordance with its well-described adjuvant functions IFNα may lead to the production of Th1 type antibodies in TLR-deficient mice [26].

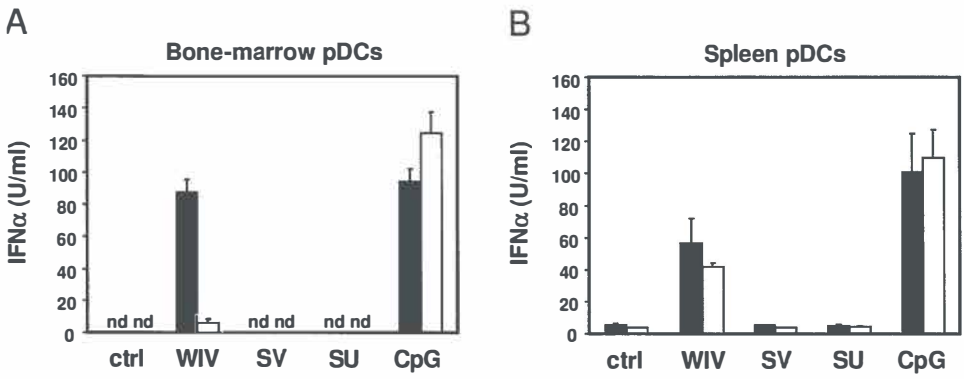


Figure 4. Induction of IFNα by WIV is TLR7-dependent in bone-marrow derived pDCs, but not in spleen-derived pDCs. *Bone-marrow cells cultured with FLT3L (containing 20–30% pDCs) (A), or pDC-enriched spleen cell cultures (containing 62%–68% pDCs) (B) of wild-type mice (black bars) and TLR7–/– mice (white bars) were incubated overnight with WIV, SV, or SU vaccine. IFNα was measured in cell supernatants by sandwich ELISA. Bars represent average values of triplicate determinations with standard deviation, and are representative of three independent experiments.*

Possible TLR-independent pathways activated by WIV may involve the retinoic acid-inducible gene*(RIG-I)* [29]–[32]. *RIG-I* is a cytoplasmic RNA-helicase that recognizes influenza virus by binding viral ssRNA bearing 5′-triphosphates which leads to IFNα production [33],[34]. The inactivated virus particles in WIV vaccine retained their membrane-fusion property (Text S2) and part of the viral genomes could therefore have entered the target cell cytoplasm to be sensed by *RIG-I*.

Taken together our observations show that the superior immune response to WIV, relative to that to SV or SU vaccines, is driven primarily by TLR-dependent mechanisms. Herein the presence of the viral RNA in the vaccine seems to play a crucial role. In contrast to SV and SU vaccines WIV contains substantial amounts of viral RNA. Removal of ssRNA from WIV by detergent solubilization and ultracentrifugation followed by reconstitution of the viral membrane envelopes to virosomes abolishes the capacity of the vaccine to induce production of IFNα by pDCs *in vitro* (Text S3 and Figure S1A) and type 1 immune responses *in vivo* [15]. On the other hand, ssRNA purified from WIV and condensed with polyethylenimine (PEI) did induce IFNα production in vitro (Text S3 and Figure S1B). Obviously, exposure of the viral RNA to β-propiolactone in the course of virus inactivation leaves the RNA intact to trigger TLR7-mediated signaling pathways (Figure 4), which translates into a strong and Th1-skewed antibody response to WIV in wild-type mice. In addition, the viral RNA may contribute to

the TLR-independent part of the response to WIV since TLR7-independent production of IFNα could only be induced in pDCs by WIV and not by formulations (SV, SU, or reconstituted viral envelopes) which lack viral RNA (Figure 4B) [15]. These lines of evidence point to the ssRNA in WIV as the key component that enhances and steers the adaptive immune response by involvement of innate immune mechanisms.

IFNα induction in pDCs clearly discriminates WIV from SV and SU vaccines but seems to occur independent of TLR7. The fact that the immune response to WIV is predominantly dependent on TLR7 then suggests that other TLR7-mediated mechanisms, possibly involving conventional DCs and B cells, critically contribute to the immune reaction. Recently, an *in vitro* study on B cells showed that TLR7 stimulation or CD40-CD40L binding by itself triggers IgG1 antibody production, but when simultaneously present induce proliferation and a switch to IgG2a production [25]. Additional stimulation of IFNα/β receptors on the same cells further drives the production of IgG2a at the expense of IgG1 antibodies [25]. Although this model might represent an over-simplification of the *in vivo* situation, it is in line with our data. The different scenarios encountered upon immunization of wild-type and mutant mice with WIV, SV, or SU are summarized in Table 2. WIV provides the ssRNA for direct triggering of TLR7 in B cells as well as the CD40 ligand for CD40 stimulation on B cells through strong T helper cell induction, which was shown also to depend on TLR7 signalling. Together with IFNα produced by TLR7-mediated and/or TLR7-independent mechanisms, these signals will lead to the enhanced and strongly polarized Th1-type antibody responses characteristic for WIV. In the absence of TLR7, WIV-induced IFNα can still stimulate moderate production of Th1 type antibodies and increase the total IgG. In contrast, SV and SU vaccines are poor inducers of T helper cells and IFNα, and cannot stimulate B cells directly via TLR7. Consequently, SV and SU vaccines induce lower and more Th2-polarized antibody responses.

Table 2. Putative vaccine effects contributing to different adaptive immune responses based on the model proposed by Heer et al. [25].

Vaccine	Mouse Strain	Direct Vaccine Effects			Result	
		IFNα Production	Th Cell Induction	TLR7-Mediated B-Cell Stimulation	Antibody Response	Phenotype
WIV	wt	+	++	+	+++	Th1
	$TLR7^{-/-}$	+	+	–	++	Th1/Th2
SV/SU	wt	–	+	–	+	Th2
	$TLR7^{-/-}$	–	+	–	+	Th2

Differences in responses induced in either wild-type or TLR7-deficient mice by WIV and SV or SU vaccine are given semiquantitatively for each of the indicated facets of the innate or adaptive response.

Our data provide mechanisms which explain the superiority of WIV vaccine to prime HA-specific immune responses in mice. Whether similar mechanisms are operational in humans and contribute to the stronger immunogenicity of WIV compared to SV or SU in unprimed individuals remains to be elucidated. Despite the favourable immunogenic properties of WIV, recent clinical trials performed in the context of pandemic vaccine development show that even with WIV at least two immunizations with a substantial amount of antigen (15–30 μg) and/or the addition of adjuvants will probably be required to achieve immune responses that comply with the CPMP criteria. If TLRs are involved in the priming of humans with WIV, their role during recall responses may be less critical, given the fact that in general WIV, SU, and SV induce similar HI titres in primed populations [11]. Use of WIV derived from wild-type virus instead of recombinant vaccine strains resulted in good antibody titres even without the addition of adjuvants and might thus be an option to obtain satisfying immune responses [35]. Evaluation of adjuvants in combination with WIV in clinical trials is so far restricted to aluminium salts. However, where adjuvanted and non-adjuvanted WIV were compared side-by-side, effects of this Th2 adjuvant on vaccine efficacy were absent, poor, or inconsistent [36]. So, better adjuvants have to be found that work synergistically with WIV in order to exploit the full potential of intact inactivated virus particles as vaccines.

In conclusion, our data reveal, for the first time to our knowledge, that TLRs play an eminent role in the immune responses to a classic influenza vaccine. Of the three influenza vaccine formulations studied here, only WIV efficiently

triggered TLR7-mediated mechanisms leading to superior immune responses. Processing of inactivated whole virus particles into SV or SU eliminates the immuno-potentiating effect of the viral ssRNA, the primary PAMP in WIV vaccine, and results in a loss of quantity and shift in the quality of the immune response. Thus, TLR-dependent mechanisms appear to form the basis for WIV's antigen-sparing quality and hence its recognized strong potential as a pandemic vaccine candidate [7],[12]. Optimizing TLR7-signalling by rational vaccine design may produce even more potent vaccines, which are urgently needed in the face of the current influenza pandemic threat.

Methods

VACCINES AND REAGENTS

H5N1 virus (NIBRG-14, a 2:6 recombinant of A/Vietnam/1194/2004 [H5N1] and A/PR/8/34 [H1N1] virus produced by reverse genetics technology) was provided by the National Institute for Biological Standards and Controls (NIBSC; Potters Bar, UK), propagated on embryonated chicken eggs, inactivated with 0.1% β-propiolactone to obtain WIV, and processed into split virus vaccine or subunit vaccine according to standard procedures [37],[38]. The haemagglutinin protein concentration in the vaccines was determined by single radial immunodiffusion (SRID) [39]. Endotoxin levels in all vaccines met the requirements of the European Pharmacopoeia standard. (If, nevertheless, contamination of endotoxin [signalling via TLR4] would have played an important role we should have observed substantial differences in the response between TLR7-deficient mice [capable of signalling via TLR4)]and MyD88/TRIF-deficient mice [deficient in all TLR-derived signalling]. However, such differences were not found for any of the vaccines.) CpG DNA (ODN D19) was purchased from Eurogentec (Seraing, Belgium).

MICE AND VACCINATION

For immunization experiments, C57BL/6, TLR7$^{-/-}$ and MyD88$^{-/-}$/TRIF$^{-/-}$ mice (generated from MyD88$^{-/-}$ mice [40] and TRIF$^{-/-}$ mice [41]) were bred at the University of Massachusetts Medical School (Worcester, MA). For *in vitro* studies, 10- to 12-week-old female C57BL/6 mice were purchased from Harlan Netherlands B.V. (Zeist, The Netherlands), and TLR7$^{-/-}$ mice (a gift from S. Akira and C. Reis e Sousa) were bred at the University Medical Center Groningen. All experiments were conducted with approval of the local Institutional Animal Care

and Use Committees. Mouse groups were matched for sex and age. Groups (n = 6–8) of C57BL/6, TLR7$^{-/-}$, and MyD88$^{-/-}$/TRIF$^{-/-}$ mice were intramuscularly injected with 50 μl of PBS in each calf muscle containing a total of 5 μg haemagglutinin protein per mouse of either WIV, SV, or SU vaccine formulation or no vaccine as a control. At 28 days after immunization, sera and spleens were collected for evaluation.

Quantitative PCR

Relative viral RNA content of the different vaccines was determined using a two-step real-time RT-PCR assay amplifying a 193-bp fragment within the M1 gene of influenza A viruses. For this purpose RNA was extracted from WIV, SV, or SU (5 μg HA) with the QIAamp viral RNA Mini Kit (QIAGEN, Venlo, The Netherlands), cDNA synthesis was performed on 5 μl of viral RNA (one-tenth of the final elution volume) using the Verso cDNA kit from ABgene (Westburg, Leusden, The Netherlands), and 1 μM UNI12 primer (5′-AGCAAAAGCAGG-3′, corresponding to viral noncoding nucleotides 1 to 12 [42]). Real-time PCR was performed with 200 nM M1-FOR primer (5′-CCTGGTATGTGCAACCTGTG-3′) and M1-REV primer (5′-AGCCTGACTAGCAACCTCCA-3′); purchased from Eurogentec, and the Absolute QPCR SYBR Green Mix (ABgene). Amplification was performed on a StepOne apparatus (Applied Biosystems), and consisted of 15 min initial activation at 95°C, followed by 40 thermal cycles of 15 sec at 95°C and 60 sec at 60°C. In each experiment, a standard curve (R^2>0.99 within the range of 1×10^2 to 1×10^9copies per reaction) was drawn to convert the respective cycle threshold (C_t) values into the number of viral genome copies. This standard consisted of a pCR2.1-TOPO plasmid construct in which was cloned a 473-bp sequence of influenza A/Puerto Rico/8/34 segment 7.

Haemagglutination inhibition assay

The HI assay was performed as described before [15]. Briefly, heat-inactivated mouse serum was absorbed to 3 volumes 25% kaolin/PBS (Sigma-Aldrich, Inc., St. Louis, MO), 20 min at room temperature (RT). After centrifugation, 50 μl of supernatant was serially diluted two-fold in a round-bottom microtitre plate (Costar, Corning Inc., Corning, NY), in duplicate. Subsequently, 50 μl PBS was added containing 2 HAU of H5N1 (NIBRG-14) virus and incubated for 40 min at RT. We used 2 HAU of virus instead of the standard 4 HAU to increase the sensitivity of the assay. Finally, 50 μl of 1% guinea pig erythrocytes (Harlan) in PBS

was added to each well and HI titres were determined after 2 h incubation at room temperature. HI titres are given as the reciprocal of the highest serum dilution producing complete inhibition of haemagglutination.

VIRUS-NEUTRALIZATION ASSAY

The levels of virus-neutralizing (VN) serum antibodies were determined with a VN assay [15],[43]. The VN titre was defined as the reciprocal of the highest serum dilution capable of inhibiting 200 TCID50 of H5N1 vaccine strain virus (NIBRG-14) from infecting Madin-Darby canine kidney cell monolayers in a microtiter plate. Infection was measured by an ELISA on intracellularly produced viral NP protein. Inhibition of infection by simultaneous incubation with mouse serum was established if the ELISA absorbance value (A_{492}) measured was below the cut-off value, determined by the equation: [(average A_{492} of the positive controls (infected cells) minus average A_{492} of the negative controls (non infected cells)) divided by 2] plus the average A_{492} of the negative controls. Serum samples were tested in quadruplicate.

ISOTYPE ELISA

Microtitre plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 0.2 μg influenza H5N1 (NIBRG-14) subunit vaccine per well in 100 μl coating buffer, overnight. After blocking with 2% milk in coating buffer for 45 min, 100 μl of two-fold serial dilutions of serum samples in 0.05%Tween 20/PBS (PBS/T) were applied to the wells and incubated for 1.5 h, in duplicate. Subsequently, 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG-isotype antibody (Southern Biotech, Birmingham, Alabama) was applied for 1 h. All incubations were performed at 37°C. Staining was performed using o-phenylene-diamine (OPD) (Eastman Kodak Company) and absorbance was read at 492 nm (A_{492}) with an ELISA reader (Bio-tek Instruments, Inc.). After subtraction of background levels, serum dilutions yielding an OD of 0.2 were calculated using linear regression, of which the reciprocal of the average of the duplicates represents the titre.

IFNγ AND IL4 ELISPOT ASSAYS

This assay was performed as described previously [15]. In short, erythrocyte-depleted splenocytes were seeded at a concentration of 5×10^5 cells in 100 μl medium per well, in triplicate in a microtitre plate (Greiner), which was pre-coated

with anti-IFNγ or anti-IL4 capture antibody (Pharmingen, San Diego, CA) and blocked with 4% BSA/PBS (Sigma-Aldrich). Cells were stimulated with 1 μg H5N1 (NIBRG-14) subunit vaccine per well, overnight in a humidified CO_2 incubator at 37°C. Cells were lysed with 100 μl of H_2O per well and plates were washed extensively, after which 100 μl of biotinylated anti-IFNγ or anti-IL4 (Pharmingen) in 2% BSA/PBS was added 1 h at 37°C. Subsequently, the plates were incubated with 100 μl of alkaline phosphatase conjugated streptavidin (Pharmingen) in 2% BSA/PBS for 1 h at 37°C, spots were visualized with 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich) substrate immobilized in solidified agarose. Plates were scanned and spots were counted manually.

PLASMACYTOID DENDRITIC CELLS

Plasmacytoid DCs were generated from bone marrow cells of C57BL/6 or TLR7$^{-/-}$ mice by seeding 1–2×10^6 bone marrow cells per well of a 24-well plate and culturing the cells for one week in Iscove's Modified Dulbecoo's Medium (IMDM) with 10% FCS and 100 ng/ml FLT3L (R&D Systems, Abingdon, UK)[22].

Single splenocyte suspensions were produced by collagenase D (Roche Diagnostics GmbH, Germany) treatment of the spleens, and spleen cell populations enriched for plasmacytoid DCs (pDCs) were obtained after magnetically labelling of pDCs with anti-mPDCA-1 antibody conjugated MicroBeads (Miltenyi Biotech GmbH, Germany) and separation over a MACS Column (Miltenyi), according to the manufacturers protocol. Percentages of pDCs in the positively selected population were determined by FACS analysis using anti-mPDCA-1-PE antibody (Miltenyi) and anti-CD11c-FITC (GeneTec Inc., Canada). Cell suspensions containing 1–2×10^5 pDCs in 100 μl were seeded in a microtitre plate and stimulated in triplicate with an equal volume containing 1.0 μg HA of either WIV, SV, or SU vaccine, or 1.0 nmol CpG DNA. After 20 h of incubation in a humidified CO_2 incubator at 37°C, supernatants were collected and subjected to the IFNα ELISA.

IFNA ELISA

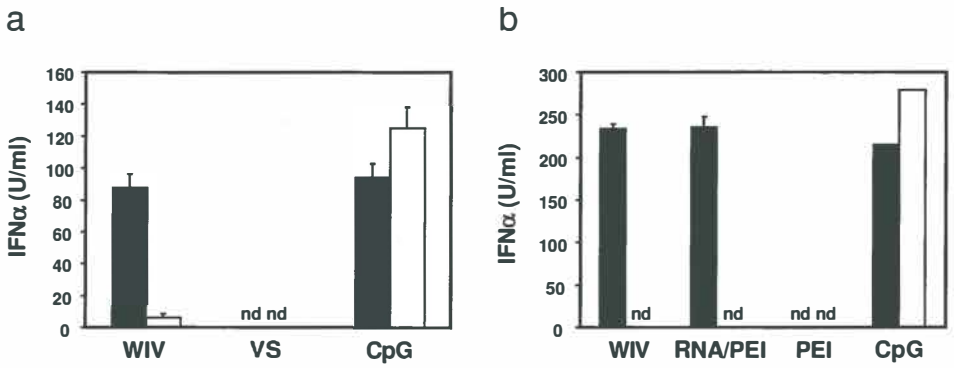
IFNα detection in cell-culture supernatants was performed using a sandwich ELISA as described previously [15]. IFNα concentrations were calculated from a recombinant IFNα (HyCult, Biotechnology, Uden, The Netherlands) standard curve performed in quadruplicate using linear regression, and expressed in units per ml.

STATISTICS

Statistical analysis on HI titres, antibody titres, and Elispot counts was performed with SPSS (SPSS 1202 Inc., Chicago, IL) using the Mann-Whitney *U* test with a CI of 95%. All *p* values are two-tailed. Statistical significance was defined as $p<0.05$.

Supporting Information

Figure S1.



Text S1.

*Calculation of relative contributions of TLR-dependent and -independent mechanisms to WIV's superior IgG response. The contribution of TLR-dependent and -independent mechanisms to the IgG response was derived using the equation: {(x-y)/(x-z)}*100% , where x is the GMT of WIV immunized wild-type mice (=42,105), y is the GMT of WIV-immunized MyD88-/-/TRIF-/- mice (=11,889) and z is the GMT of SV-immunized wild-type mice (=876) or, when compared to SU vaccine, the GMT of SU-immunized wild-type mice (=5,481).*

Text S2.

H5N1 virus inactivated with β-propiolactone is fusion-active. Fusion activity of H5N1 virus (NIBRG-14), inactivated with 0.1% β-propiolactone, was evaluated with a haemolysis assay and a fluorescence membrane fusion assay using WIV labelled with octadecyl Rhodamine B (R18), as described previously (Stegmann T, et al. (1993) Biochemistry 32: 11330-11337). The experimental haemolysis of human erythrocytes by WIV, as a percentage of maximal haemolysis by water treatment of the erythrocytes, was 45.2% at pH 5.5. Comparatively, haemolysis values for split virus and subunit in the same test were 0.3% and 0.5% respectively. Fusion activity, measured with the R18 assay, ranged between 25% and 30% at pH 5.5 for WIV.

Text S3.

Viral RNA from β-propiolactone-inactivated WIV vaccine stimulates TLR7.Virosomes (VS) were prepared from β-propiolactone-inactivated H5N1 virus (NIBRG-14), by solubilization of the viral membrane, followed by removal of the nucleocapsid by ultracentrifugation, and subsequent reconstitution of the viral membrane envelope, as described previously (De Jonge J, et al. (2006) Biochimica et Biophysica Acta 1758: 527-536). Viral RNA was isolated from H5N1 WIV preparations using a RNeasy Kit (QIAGEN, Venlo, The Netherlands) and condensed with polyethylenimine (PEI, Sigma-Aldrich, Inc., St. Louis, MO), as described elsewhere (Diebold SS, et al. (2004) Science 303: 1529-1531). Stimulation experiments were performed as described in the Methods section of the manuscript. In short, bone-marrow derived pDC cultures were stimulated with WIV or VS in a concentration of 5 μg HA per ml, or RNA/PEI complexes in a concentration of 1 μg RNA per ml, or PEI alone, or CpG DNA. The IFNα response is shown in Figure S1. Virosomes (VS) which are virus-like particles devoid of RNA do not induce detectable (nd) IFNα production in pDCs from wild-type mice (black bars) or TLR7-/- mice (white bars) (Fig. S1A). In contrast RNA/PEI complexes induce IFNα production in pDCs from wild-type mice (black bars) but not from TLR7-/- mice (white bars) (Fig. S1B). These experiments confirm that it is the viral genomic RNA in WIV that activates TLR7-mediated pathways, as was shown by others (Diebold SS, et al. (2004) Science 303: 1529-1531), and that the inactivation procedure with β-propiolactone has no effect on the ability of the viral genome to stimulate TLR7.

Acknowledgments

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References

1. Subbarao K, Klimov A, Katz J, Regnery H, Lim W, et al. (1998) Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279: 393–396.
2. WHO (2008) Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO. Available: http://www.who.int/csr/disease/avian influenza/country/cases table 2008 06 19/en/index.html. Accessed 10 July 2008.
3. Poland GA, Jacobson RM, Targonski PV (2007) Avian and pandemic influenza: An overview. Vaccine 25: 3057–3061.
4. WHO (2006) Department of Immunization, Vaccines and Biologicals. Global pandemic influenza action plan to increase vaccine supply, p. 4. Available: http://www.who.int/vaccines-documents/DocsPDF06/863.pdf. Accessed 13 December 2007.
5. Stephenson I (2006) H5N1 vaccines: How prepared are we for a pandemic? Lancet 368: 965–966.
6. WHO (2005) Development and evaluation of influenza pandemic vaccines: Report of WHO expert meeting, Initiative for Vaccine Research, Global Influenza Programme. Available:http://www.who.int/vaccine research/documents/Report%20021105.pdf. Accessed 13 December 2007.
7. Lin J, Zhang J, Dong X, Fang H, Chen J, et al. (2006) Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: A phase I randomised controlled trial. Lancet 368: 991–997.
8. Wood JM, Williams MS (1998) History of inactivated influenza vaccines. In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of influenza. Oxford: Blackwell Science. pp. 317–323.
9. Nicholson KG, Tyrrell DA, Harrison P, Potter CW, Jennings R, et al. (1979) Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: Serological responses and clinical reactions. J Biol Stand 7: 123–136.
10. Wright PF, Thompson J, Vaughn WK, Folland DS, Sell SH, et al. (1977) Trials of influenza A/New Jersey/76 virus vaccine in normal children: an overview of age-related antigenicity and reactogenicity. J Infect Dis 136: S731–S741.
11. Beyer WE, Palache AM, Osterhaus AD (1998) Comparison of serology and reactogenicity between influenza subunit vaccines and whole virus or split vaccines: A review and meta-analysis of the literature. Clin Drug Investig 15: 1–12.
12. Stephenson I, Nicholson KG, Glück R, Mischler R, Newman RW, et al. (2003) Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: Phase I randomised trial. Lancet 362: 1959–1966.

13. McLaren C, Verbonitz MW, Daniel S, Grubbs GE, Ennis FA (1977) Effect of priming infection on serologic response to whole and subunit influenza virus vaccines in animals. J Infect Dis 136: S706–S711.

14. Hovden AO, Cox RJ, Haaheim LR (2005) Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol 62: 36–44.

15. Geeraedts F, Bungener L, Pool J, Ter Veer W, Wilschut J, et al. (2008) Whole inactivated virus influenza vaccine is superior to subunit vaccine in inducing immune responses and secretion of proinflammatory cytokines by DCs. Influenza Other Respir Viruses 2: 41–51.

16. Bungener L, Geeraedts F, Ter Veer W, Medema J, Wilschut J, et al. (2008) Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine 26: 2350–2359.

17. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783–801.

18. Medzhitov R, Janeway CA Jr (1997) Innate immunity: Impact on the adaptive immune response. Curr Opin Immunol 9: 4–9.

19. Pasare C, Medzhitov R (2004) Toll-like receptors and acquired immunity. Semin Immunol 16: 23–26.

20. Guay HM, Andreyeva TA, Garcea RL, Welsh RM, Szomolanyi-Tsuda E (2007) MyD88 is required for the formation of long-term humoral immunity to virus infection. J Immunol 178: 5124–5131.

21. Gavin AL, Hoebe K, Duong B, Ota T, Martin C, et al. (2006) Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science 314: 1936–1938.

22. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303: 1529–1531.

23. Snapper CM, Paul WE (1987) Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 236: 944–947.

24. Jouvin-Marche E, Morgado MG, Leguern C, Voegtle D, Bonhomme F, et al. (1989) The mouse Igh-1a and Igh-1b H chain constant regions are derived from two distinct isotypic genes. Immunogenetics 29: 92–97.

25. Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, et al. (2007) TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. J Immunol 178: 2182–2191.

26. Proietti E, Bracci L, Puzelli S, Di Pucchio T, Sestili P, et al. (2002) Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. J Immunol 169: 375–383.

27. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, et al. (2001) Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity 14: 461–470.

28. Hochrein H, Schlatter B, O'Keeffe M, Wagner C, Schmitz F, et al. (2004) Herpes simplex virus type-1 induces IFN-alpha production via Toll-like receptor 9-dependent and -independent pathways. Proc Natl Acad Sci U S A 101: 11416–11421.

29. Barchet W, Krug A, Cella M, Newby C, Fischer JA, et al. (2005) Dendritic cells respond to influenza virus through TLR7- and PKR-independent pathways. Eur J Immunol 35: 236–242.

30. López CB, Moltedo B, Alexopoulou L, Bonifaz L, Flavell RA, et al. (2004) TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses. J Immunol 173: 6882–6889.

31. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, et al. (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5: 730–737.

32. Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, et al. (2007) Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol 179: 4711–4720.

33. Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, et al. (2006) 5′-Triphosphate RNA is the ligand for RIG-I. Science 314: 994–997.

34. Pichlmair A, Schulz O, Tan CP, Näslund TI, Liljeström P, et al. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science 314: 997–1001.

35. Kistner O (2007) Phase 1/2 clinical study with Baxter's H5N1 vaccine: Clinical update. WHO meeting on evaluation of pandemic influenza prototype vaccines in clinical trials, Geneva, Feb 15–16, 2007. Available: http://www.who.int/vaccine research/diseases/influenza/150207 Kistner Baxter.pdf. Accessed 25 March 2008.

36. World Health Organization. Tables on the clinical trials of pandemic influenza prototype vaccines. Available: http://www.who.int/vaccine research/diseases/influenza/flu trials tables/en/index3.html. Accessed 25 March 2008.

37. Fenters JD, Yamashiroya HM, Petzold RF, Tolkacz VK (1970) Enhanced immunogenicity in mice of a purified, tween-ether-treated influenza vaccine. Appl Microbiol 20: 544–550.

38. Brady MI, Furminger IG (1976) A surface antigen influenza vaccine. 1. Purification of haemagglutinin and neuraminidase proteins. J Hyg (Lond) 77: 161–172.

39. Williams MS, Mayner RE, Daniel NJ, Phelan MA, Rastogi SC, et al. (1980) New developments in the measurement of the hemagglutinin content of influenza virus vaccines by single-radial-immunodiffusion. J Biol Stand 8: 289–296.

40. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, et al. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 9: 143–150.

41. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, et al. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301: 640–643.

42. Lamb RA, Choppin PW (1983) The gene structure and replication of influenza virus. Annu Rev Biochem 52: 467–506.

43. Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, et al. (1999) Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol 37: 937–943.

Effect of viral membrane fusion activity on antibody induction by influenza H5N1 whole inactivated virus vaccine

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Abstract

Whole inactivated virus (WIV) influenza vaccines are more immunogenic in unprimed individuals than split-virus or subunit vaccines. In mice, this superior immunogenicity has been linked to the recognition of the viral ssRNA by endosomal TLR7 receptors in immune cells, leading to IFNα production and Th1-type antibody responses. Recent data suggest that viral membrane fusion in target cell endosomes is necessary for TLR7-mediated IFNα induction. If so, virus inactivation procedures that compromise the fusion activity of WIV vaccines, like formaldehyde (FA) treatment, could potentially harm vaccine efficacy. Therefore, we measured the effect of fusion inactivation of H5N1 WIV on TLR7 activation *in vitro*, and on antibody isotype responses *in vivo*. Fusion inactivation of WIV reduced, but did not block, TLR7-dependent IFNα induction in murine dendritic cells *in vitro*. *In vivo*, fusion-inactive WIV was as potent as fusion-active WIV in inducing total H5N1-specific serum IgG and IgG2c subtype antibodies in unprimed mice. Both vaccines induced only small amounts of IgG1. However, FA treatment of WIV did reduce the capacity of the vaccine to induce hemagglutination-inhibiting (HI) antibodies. This possibly relates to modification of epitopes that are targets for HI antibodies rather than to loss of fusion activity. Antibody affinity maturation was not negatively affected by fusion inactivation. In conclusion, fusion activity of H5N1 WIV does not play a major role in Th1-type antibody induction. Yet, to preserve the full immunogenicity of WIV, or possibly also other inactivated influenza vaccines, harsh treatment with formaldehyde should be avoided.

Introduction

Aside from yearly epidemics, influenza A viruses occasionally cause pandemics, leading to excess morbidity and deaths. Pandemics, at least those in the recent past, resulted from the introduction of a new influenza virus originating from birds or swine into the human population [1-5]. Avian viruses that incidentally infect humans like highly pathogenic H5N1, H7N7, and H9N2 present a pandemic risk, as these viruses might adapt and become transmissible from human-to-human [6-9]. This potential pandemic threat urges the development of highly efficacious influenza vaccines [10].

Influenza vaccines containing whole inactivated virus (WIV) particles induce stronger immune responses in immunologically naive individuals than split-virus or subunit vaccines [11-13]. This feature allows for dose-sparing immunization regimens without the requirement for an adjuvant [14]. WIV therefore is a promising pandemic vaccine candidate [15]. Additionally, in mice, WIV uniquely induces a Th1-type response, characterised by high levels of IgG2a/c antibodies, which correlates better with protection than a Th2-type response, as typically induced by (unadjuvanted) split-virus or subunit vaccines [16-19]. Recently, a mock-up licensure was granted by the European Medicines Agency (EMA) for an H5N1 WIV formulation, which allowed fast-track approval of an H1N1 WIV vaccine, deployed during the H1N1 flu pandemic in 2009 [20,21].

In mice, the superior immunogenicity of WIV is largely due to activation of Toll-like receptor 7 (TLR7) in immune cells by viral single-stranded (ss) RNA present in the inactivated viral particles [22,23]. In addition, TLR7 activation may also play a role in antibody affinity maturation, and long-term persistence of antigen-specific B-cell and T-cell responses [25]. It is likely that multiple TLR7-dependent mechanisms act together to produce the Th1-type antibody response induced by WIV [23,24,26-29]. TLR7 activation in B-cells on itself stimulates antibody production *in vitro*, and TLR signalling in B-cells has been shown essential for augmenting *in vivo* antibody responses [24,25]. TLR7 is also involved in the induction of CD4+ Th-cells [23,29] and IFNα production by plasmacytoid dendritic cells (pDC) [27,28]. Th-cells provide CD40L, which together with IFNα presents additional signals to activated B-cells, necessary to augment and steer the antibody production to predominantly IgG2a *in vitro* and *in vivo* [24-26].

It has been shown that TLR7-mediated IFNα production by pDCs is strongly reduced when endosomal acidification is blocked [27,28]. The low

endosomal pH is the natural trigger for viral membrane fusion and uncoating, and it has been suggested that these viral activities are important for TLR7 signalling [30]. This implicates that treatments which affect the membrane fusion capacity of WIV, for instance virus inactivation protocols that use formaldehyde [31], could affect the vaccine's capacity to trigger TLR7 and therefore impair the immunogenicity of the vaccine. To study the role of viral membrane fusion activity in the immunogenicity of WIV, we used a formaldehyde treatment protocol to inactivate the fusion activity of WIV particles, and measured the effect of this treatment on particle uptake and TLR7 activation in dendritic cells *in vitro* and on antibody subtype responses *in vivo*.

Materials and methods

VIRUS, VIRUS INACTIAVTION, AND FUSION INACTIVATION

H5N1 virus (NIBRG-14, provided by NIBSC, Potters Bar, UK) was propagated on eggs. Egg-derived H3N2 virus strain A/Panama/2007/99 was kindly provided by Solvay Biologicals, Weesp, The Netherlands. WIV vaccine was produced by using 0.1% β-propiolactone (BPL) to inactivate influenza virus replication [23]. The amount of HA and total viral protein in the WIV vaccine stock was 0.99 μg/μl and 9.88 μg/μl, as determined by single radial immunodiffusion (SRID) and micro Lowry assay, respectively [23,52]. To inactivate the fusion activity of WIV, purified viral particles were incubated with formaldehyde (FA) using conditions as indicated under Results. Formaldehyde treatment was followed by immediate dialysis against HNE buffer (5 mM Hepes, 0.15 M NaCl, 0.1 mM EDTA, pH 7.4).

MICE AND IMMUNIZATION

TLR7-/- mice (a kind gift from Dr. S. Akira and Dr. C. Reis e Sousa) were bred at the animal facility of the University Medical Center Groningen and were used for isolation of bone marrow and subsequent culture of bone-marrow-derived dendritic cells (BMDC) [32]. Female C57Bl/6 mice, 6-8 weeks old, were obtained from Harlan (The Netherlands). Mouse experiments were conducted with approval of the local institutional committee for animal care and use. Mice were immunized intramuscularly with 1 or 5 μg of fusion-active or fusion-inactive H5N1 WIV in HNE buffer. Four weeks after immunization, mice were sacrificed and blood was collected.

DENDRITIC CELLS

BMDC cultures, enriched for plasmacytoid dendritic cells (pDC), were established as described by Gilliet *et al.* [33] with minor modifications [23]. After 8 days of culture, cells were harvested and analysed for pDC's using anti-PDCA-1-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD11c-FITC (Genetex Inc, CA, USA) staining and flow-cytometric analysis.

FUSION ASSAYS

The fusion capacity of WIV was measured by octadecylrhodamine (R18) dequenching after fusion of R18-labeled WIV with eryhtrocyte ghost membranes and by a hemolysis assay. R18-labeled WIV was prepared by adding R18 (Molecular Probes, Eugene, Oregon, USA) in ethanol to WIV at an amount of 9 mol % of the phospholipid content of WIV (determined by a phosphate assay [53]),followed by incubation at room temperature (RT) for 1 hr in the dark. Free R18 was separated from viral membrane-bound R18 by gel filtration chromatography on a Sephadex G75 column (Pharmacia, Sweden) and labeled virus was collected. The capacity of R18-labeled WIV to fuse with eryhtrocyte ghost membranes was investigated and calculated as previously described [34]. Fusion activity of WIV determined by hemolysis was determined as described before [35].

IN VITRO UPTAKE OF FUSION-ACTIVE AND FUSION-INACTIVE WIV BY BMDC

To study the uptake of fusion-active and fusion-inactive WIV by dendritic cells, WIV preparations were labeled with fluorescein-isothiocyanate (FITC, Sigma), as described previously [36]. FITC-labeled fusion-active or FITC-labeled fusion-inactive WIV was then incubated at concentrations of 0.1, 1, and 10 μg viral protein per ml, with BMDC for 30 min at 37° in 15 ml polypropylene tubes. Next, cells were placed on ice and 2.5 μg/ml of neuraminidase (Clostridium-derived, Type V, Sigma) was added to remove bound virus from the cell surface. After incubation for 1 hr, cells were washed in ice-cold serum free IMDM, incubated for 20 min with anti-CD11c-PE-Cy5 (eBioscience, San Diego, USA) and washed with PBS. Cells were then fixed by a 10 min incubation in FACS lysing solution (BD). After a final wash in PBS/0.02% BSA, cells were analysed on a FacsCalibur (BD) flow cytometer.

IN VITRO INDUCTION OF IFNA IN WIV-STIMULATED BMDC

Fusion-active and fusion-inactive WIV in different concentrations, or 1.0 nmol CpG DNA (D19; GGTGCATCGATGCAGGGGGG; Eurogentec, Belgium), was added to triplicate wells of BMDCs (1–2×10^5 BMDC/well) in 96 wells plates. Culture supernatants were harvested 16 hr later and assayed for IFNα using an IFNα ELISA as described before [19, 23].

ANTIBODY IGG SUBTYPE ELISA, HEMAGGLUTINATION-INHIBITION ASSAY AND AFFINITY MEASUREMENTS

For detection of virus-specific serum antibodies of different subtypes, sera of immunized mice were tested by ELISA, as described before [23]. The presence of hemagglutination-inhibiting (HI) antibodies were determined by employing a standard HI assay [19, 23]. To allow a comparison of affinity of H5N1-specific IgG antibodies, pooled serum samples from immunized mice were first diluted such that they contained comparable levels of H5N1-specific IgG. The sera were then incubated in H5N1-coated ELISA plates for 1.5 hr. After washing with PBS/T, separate sets of wells with bound antibody were washed with different concentrations (ranging from 0.125 to 4 M) of ammoniumthiocyanate (NH_4SCN) in phosphate buffer (pH6.0) for 15 min at RT. [37] After three washes with PBS/T, the remaining amount of antibody still bound in the antigen-coated wells was assessed using horseradish peroxidase-conjugated goat anti-mouse IgG as indicated above and expressed as OD at 492 nM.

STATISTICAL ANALYSIS

Statistical analysis was performed using Graphpad Prism 5.0 (Graphpad software Inc., La Jolla, CA, USA).

Results

FUSION INACTIVATION

Fusion-active WIV caused lysis of 60% to 83% of human erythrocytes in the hemolysis assay, in case of H5N1 WIV or H3N2 WIV, respectively, at the optimal pH for membrane fusion (pH5.5 for H5N1, and pH5.2 for H3N2). Viral membrane fusion activity was completely abrogated after incubation of the WIV preparation with 0.5% FA for 30 min at 37°C (Fig 1A). The loss of fusion activity by treatment

with 0.5% FA was confirmed in a fluorometric assay. In this assay, untreated R18-labelled H3N2 WIV at a pH of 5.2 showed rapid fusion with erythrocyte ghosts, up to a final extent of 75%, while fusion activity was completely abolished after treatment of the preparation with 0.5% FA (Fig 1B). Treatment with 0.02% FA required a longer incubation time to result in complete fusion inactivation (data not shown).

The influence of fusion inactivation on the uptake of viral particles by immune cells was assessed by incubating BMDC (>85% CD11C+) with H5N1 WIV, labelled with FITC (10 μg viral protein /ml), followed by FACS analysis. BMDC showed similar levels of uptake of fusion-active and fusion-inactive particles, as evidenced by similar mean fluorescence intensities of BMDC incubated with the different WIV preparations (Fig 1C). Incubation with lower concentrations of WIV (1 and 0.1 μg viral protein /ml) also showed similar levels of uptake for both WIV preparations (data not shown). This indicates that fusion-active and fusion-inactive particles have an equal capacity to bind to and enter DCs.

IFNA INDUCTION BY FUSION-ACTIVE AND FUSION-INACTIVE WIV

The effect of fusion inactivation on TLR7 signalling was assessed by measuring the IFNα produced by BMDC after incubation with WIV. In wt BMDC fusion-active and fusion-inactive H5N1 WIV induced IFNα in a dose-dependent manner (Fig 2A). Amounts of IFNα produced upon incubation with 5 μg/ml fusion-inactive WIV were lower than after incubation with fusion-active WIV ($p<0.05$, in a one-sided Mann-Whitney U test). At 0.5 μg/ml both formulations induced equal amounts of IFNα. (Fig 2A). IFNα production was absent in TLR7-/- BMDC stimulated with fusion-active and fusion-inactive WIV, which shows that the IFNα induction in wt BMDC by both vaccines is completely TLR7 dependent (Fig 2B).

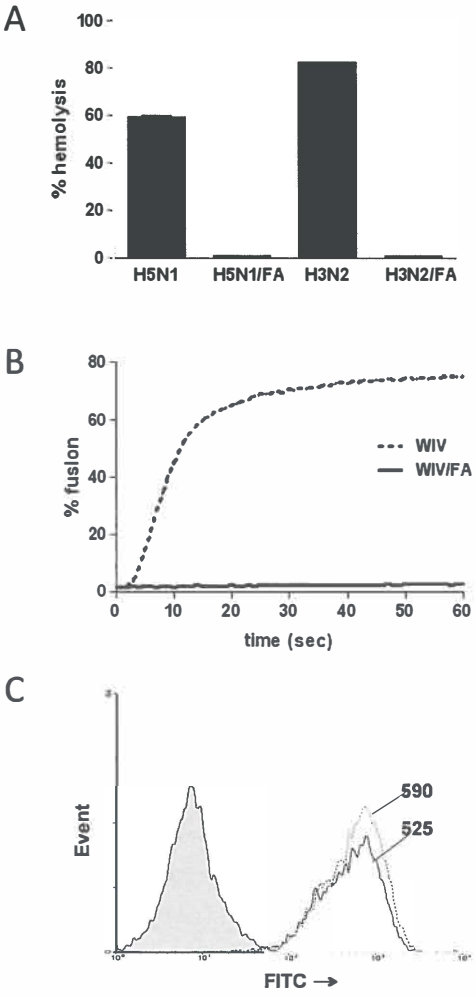

Figure 1. Inactivation of viral membrane fusion activity. *WIV was treated with buffer alone (WIV) or with buffer containing 0.5% formaldehyde (WIV/FA), for 30 min. (A) Fusion activity of WIV and WIV/FA, measured by its capacity to lyse erythrocytes, is given as a percentage of the maximal lysis, determined by hypotonic lysis of the erythrocytes in water. Results are shown for two different virus strains: H5N1 and H3N2, untreated (H5N1, H3N2) and treated with FA (H5N1/FA, H3N2/FA). (B) Fusion activity, measured by an R18-based fluorometric assay. Given is the percentage of R18-labelled H3N2 WIV particles, either untreated (dashed line) or FA-treated (solid line), that fuse with erythrocyte ghosts, plotted against the time elapsing after installing the optimal pH (pH 5.2) for triggering viral membrane fusion. (C) The binding and uptake of FITC-labelled fusion-active WIV and fusion-inactive WIV/FA by BMDC is presented by the fluorescence intensity (dashed line, WIV; solid line, WIV/FA) with the mean fluorescence intensity (MFI) given. The grey histogram shows the fluorescence intensity of BMDC incubated with medium alone. The data shown are representative results of two independent experiments.*

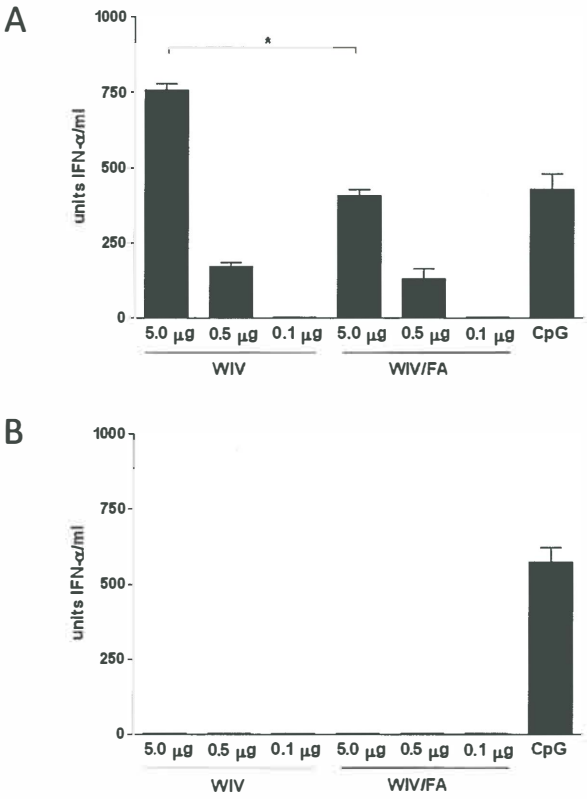

Figure 2. Induction of IFNα production in dendritic cells by fusion-acitve and fusion-inactive WIV. *BMDC cultures of wt mice (A) and TLR7-/- mice (B) enriched for pDC (containing, respectively, 30% and 27% of pDC expressing both CD11c+ and PDCA-1+) were stimulated with different amounts of fusion-active (WIV) or fusion-inactive (WIV/FA) H5N1 viral particles. After incubation for 16 hr, the IFNα concentration in the culture supernatants was measured by ELISA. Representative data from 1 of 3 individual experiments is shown. Error bars indicate the standard deviation of triplicate determinations. A value of $p<0.05$ was considered as statistically significant and is indicated in the figures with an asterisk.*

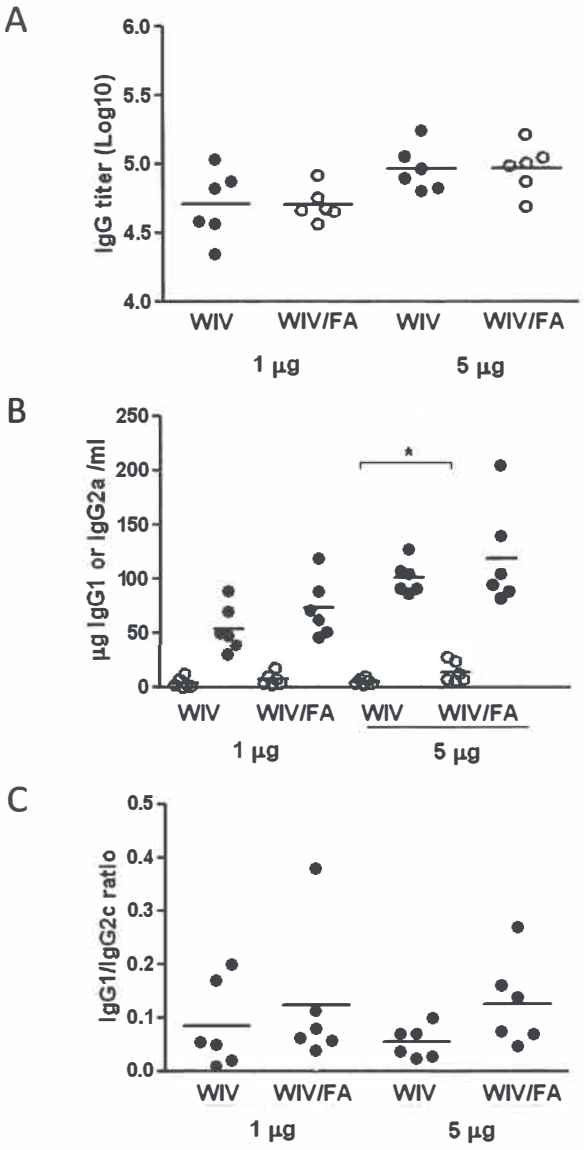

Figure 3. IgG and IgG subtype responses induced by fusion-active WIV and fusion-inactive WIV. *Mice were vaccinated once i.m. with fusion-active WIV or fusion-inactive WIV (WIV, WIV/FA, respectively; 1 µg or 5 µg HA). Serum H5N1-specific IgG (A), and IgG1 and IgG2c subtype antibodies (B) were measured by ELISA four weeks later. Concentrations of H5N1-specific serum IgG1 (open circles) and IgG2c (filled circles) were determined using subtype standards (B), and used to calculate the IgG1/IgG2c ratio (C). Horizontal lines indicate mean titer, concentration or ratio per group, respectively. A value of $p<0.05$ was considered as statistically significant and is indicated in the figures with an asterisk.*

IMMUNIZATION OF MICE WITH FUSION-ACTIVE OR FUSION-INACTIVE WIV

The *in vivo* immunogenicity of fusion-active and fusion-inactive H5N1 WIV was evaluated by intramuscular immunization of C57BL/6 mice with either type of vaccine, and analysis of the antibody response four weeks later. Serum H5N1-specific total IgG titers induced by fusion-inactive WIV did not differ from those induced by fusion-active WIV for each vaccine dose used (Fig 3A). Also, no difference was found in the concentrations of H5N1-specific IgG1 and IgG2c subtype antibodies between mouse groups immunized with fusion-active or inactive WIV, except for a small increase in IgG1 in the group immunized with 5 μg fusion-inactive WIV (Fig 3B; $p=0.04$). The IgG1/IgG2c ratios in all immunization groups were reminiscent of a strongly Th1-skewed immune response (Fig 3C).

Next to the skewing towards Th1-signature antibody production, TLR activation may also contribute to antibody affinity maturation. Because fusion-inactive WIV tended to induce lower levels of (TLR7- mediated) IFNα *in vitro*, we compared the affinity of serum antibodies in mice immunized with fusion-active WIV and fusion-inactive WIV by ELISA. In this ELISA, wells containing antigen-bound antibody were washed with different concentrations of the chaotropic agent ammonium thiocyanate (NH_4SCN). Antibodies with lower affinity will more easily be eluted with increasing concentrations of NH_4SCN than higher-affinity antibodies, resulting in lower OD values in the ELISA. Antibodies induced by fusion-inactive WIV did not show lower OD values at increasing concentrations of NH_4SCN than antibodies induced by fusion-active WIV (Fig 4). Therefore, fusion inactivation did not appear to negatively affect antibody affinity maturation *in vivo*.

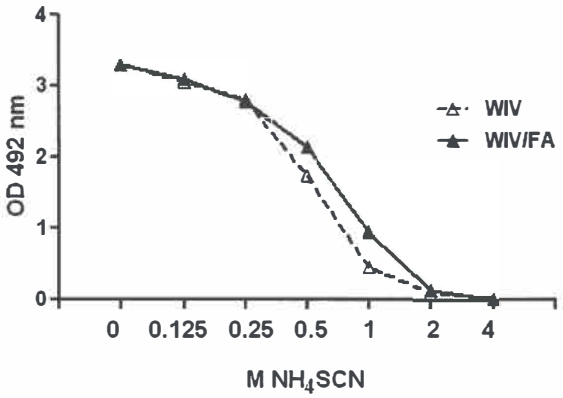

Figure 4. Effect of fusion-inactivation on antibody affinity maturation. *Antibody affinity was assessed using increasing concentrations of a chaotropic agent (NH_4SCN) to break antigen-antibody bonds of serum IgG bound to H5N1-coated ELISA plates. Plotted are the optical densities (OD) and standard deviation of triplicate determinations for pooled serum samples of mice immunized with fusion-active WIV (dashed line, WIV) or fusion-inactive FA-treated WIV (black line, WIV/FA), against the concentration of NH_4SCN in the washing buffer. The OD represents the amount of IgG that remained bound to the H5N1 antigens.*

Despite the similar ELISA antibody titers for sera from mice immunized with fusion-active WIV or fusion-inactive WIV, HI titers were clearly lower in mice immunized with fusion-inactive WIV (Fig 5A). A possible cause for this discrepancy may be the modification of epitopes on the HA by the FA treatment, leading to the induction of antibodies that do not recognize native HI epitopes. Indeed, when FA-treated viral particles were used for hemagglutination instead of untreated particles in the HI assay, a more than two-fold apparent increase in HI titer was observed for the sera from mice immunized with fusion-inactive WIV (Fig 5B). This suggests that the 0.5% FA treatment modifies epitopes on HA that are targets for HI antibodies, and that the subsequent reduction in HI titer is probably not due to loss of viral membrane fusion activity.

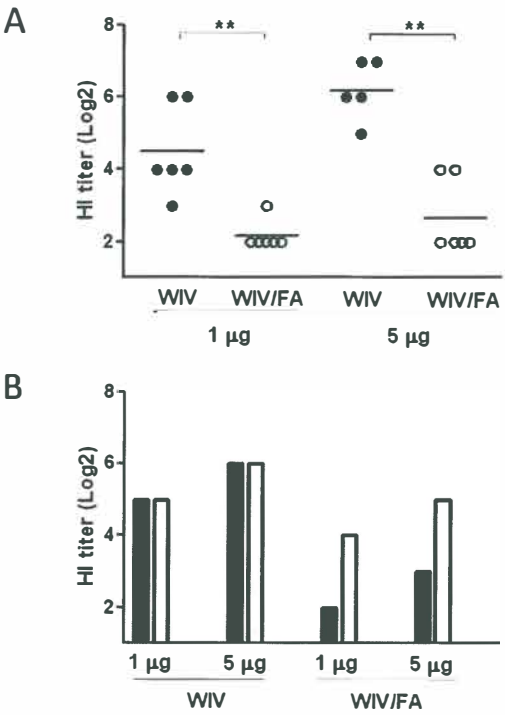

Figure 5. Serum HI titers induced by fusion-active and fusion-inactive WIV. *(A) The capacity of sera from mice immunized with fusion-active WIV or fusion-inactive WIV (WIV, WIV/FA, respectively; 1 µg or 5 µg HA) to prevent hemagglutination of guinea pig erythrocytes is given for the individual mice, and presented as HI titer. (B) To analyse possible epitope changes mediated by FA treatment, pooled serum samples from mice immunized with either fusion-active or fusion-inactive WIV (WIV, WIV/FA, respectively) were tested in an HI assay. In this assay, untreated viral particles (black bars) or FA-treated viral particles (white bars) were used to agglutinate erythrocytes. Statistically significant differences are indicated in the figure with asterisks ($p<0.01$).*

Discussion

In this study, we investigated the effect of viral membrane fusion activity on the immunological properties of influenza A/H5N1 WIV vaccine. We found that inactivation of viral membrane fusion had no effect on the uptake of WIV particles by antigen-presenting cells and a modest effect on TLR7-mediated IFNα production by DCs. *In vivo*, fusion inactivation of WIV did not have a major effect on the capacity of WIV to induce anti-HA antibodies as measured by ELISA. Induction of HI antibodies was reduced by fusion inactivation of WIV, but this

may be explained by a modifying effect of FA on HA epitopes, rather than a loss of membrane fusion activity.

We previously reported that TLR7 signalling plays an important role in the stimulation of the immune response to influenza WIV [23]. Wang and coworkers earlier postulated a role for viral membrane fusion and virus uncoating in TLR7 signalling [30]. Their hypothesis was based on the observation that prevention of fusion of (influenza) virus with the target cell endosomal membrane by raising the endosomal pH, resulted in a strong reduction of TLR7 signalling. We were able to study the role of membrane fusion in a direct way by selectively eliminating the fusion capacity of the virus particle itself, rather than by modifying the endosomal pH. We destroyed viral membrane fusion activity of WIV by a short treatment with 0.5% FA. Other methods that can inhibit the fusion activity of WIV include low-pH pretreatment, or fusion-blocking antibodies. Low-pH treatment, however, induces conformational changes in the HA affecting its receptor binding capacity or change the exposure of certain antibody epitopes for B-cell recognition. Fusion-blocking antibodies could alter the binding and entry of WIV into DC through binding to Fc-receptors. The latter may lead to enhanced uptake and TLR7 signalling (unpublished results), as has been shown previously for antibody-opsonized Coxsackie B virus [49].

Using FA treatment to inhibit the fusion activity of WIV, we found a lower IFNα production by fusion-inactive WIV, but only at the highest particle concentration. In addition to its effects on fusion activity, FA treatment may also stabilize virus particles against enzymatic degradation in celluar endosomes, or cross-link the viral RNA to nucleocapsid proteins, which could hamper the release of ssRNA for TLR7 recognition [43,44]. Such effect could explain the diminished IFNα production after FA treatment. Regarding the link between endosomal acidification and TLR7 activation demonstrated by others, our results suggest that other processes that depend on a mildly acidic endosomal pH, like viral uncoating and/or endosomal maturation [45], may prove of greater significance for TLR7 signalling than viral membrane fusion. Endosomal acidification and maturation have been proven important for TLR7 activation by synthetic TLR7 ligands, showing that target cell entry of the virus may not be the only pH-dependent process involved in the control of TLR7 activation [48].

In the absence of fusion activity, WIV maintained its capacity to induce normal IgG levels with a predominance of IgG2a/c subtype antibodies *in vivo*. This is in line with the relatively preserved capacity of WIV to activate TLR7 and

to induce IFNα production *in vitro*. Both TLR7 activation and IFNα act as signals that drive class-switch recombination towards IgG2a/c production in B cells, as was recently demonstrated in a study investigating *in vitro* and *in vivo* B-cell activation by influenza [24]. Furthermore, we did not find evidence that fusion inactivation compromises the induction of H5N1-specific IFNγ-producing cells (data not shown), suggesting that also T-cell help to B-cells remains intact.

TLR stimulation has been shown to be important for antibody affinity maturation in induction of protective antibodies to respiratory syncytial virus (RSV) [46]. Whether a similar dependency on TLR plays a role in affinity maturation of the antibody response to influenza virus remains to be clarified. Immunization of mice with nanoparticles containing HA, combined with nanoparticles containing a TLR7 ligand, yielded antibodies of higher avidity than with nanoparticles containing HA alone [25]. This clearly suggests that TLR7 could play a role in the affinity maturation of WIV-induced antibodies. We observed no negative effect of fusion inactivation on antibody affinity, which is in line with the notion that the capacity to induce TLR7 signaling was not critically affected by fusion inactivation. Taken together, these results suggest that the critical determinants that shape the antibody response to WIV are relatively unaffected by inhibition of viral membrane fusion.

WIV can induce cytotoxic T-cells (CTL), and it has recently been shown that, compared to untreated WIV, WIV treated with 0.5% FA is less efficient in priming naïve $NP_{366-374}$-specific cytotoxic T-cells (CTL) *in vivo*, but not in reactivation of memory CTL *in vitro* [50]. Fusion activity therefore might play a role in priming of CTL responses. CTLs induced by cross-priming are known to contribute to protection against heterosubtypic virus infection [51]. This could be favorable especially in a pandemic situation, when there is a chance that stockpiled vaccines do not completely match the pandemic virus.

In conclusion, viral membrane fusion is not a prerequisite for TLR7 signalling, and does not have a major effect on IFNα induction and the Th1-type skewing of antibody responses. Although, care should be taken when using FA as an agent to inactivate viral particles for WIV production as it might affect the induction of HI antibodies, it is unlikely that loss of fusion activity is a cause of impairment of vaccine efficacy.

Acknowledgements

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References

1. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. Nature. 2005;437(7060):889-93.
2. Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol. 1989;63(11):4603-8.
3. Webster RG, Laver WG. The origin of pandemic influenza. Bull World Health Organ. 1972;47(4):449-52.
4. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, López-Gatell H, Olivera H, López I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD Jr, Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 2009;325(5937):197-201.
5. Guan Y, Vijaykrishna D, Bahl J, Zhu H, Wang J, Smith GJ. The emergence of pandemic influenza viruses. Protein Cell. 2010;1(1):9-13.
6. http://www.who.int/csr/disease/avian influenza/country/ cases table 2011 01 20/en/ index.html
7. [De Wit E, Fouchier RA. Emerging influenza. J Clin Virol. 2008;41(1):1-6.
8. Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, Orr WK, Shortridge KF. Human infection with influenza H9N2. Lancet. 1999;354(9182):916-7.
9. Fedson DS. Vaccine development for an imminent pandemic: why we should worry, what we must do. Hum Vaccin. 2006;2(1):38-42.
10. Wright PF. Vaccine preparedness--are we ready for the next influenza pandemic? N Engl J Med. 2008;358(24):2540-3.
11. Nicholson KG, Tyrrell DA, Harrison P, Potter CW, Jennings R, Clark A, Schild GC, Wood JM, Yetts R, Seagroatt V, Huggins A, Anderson SG. Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. J Biol Stand. 1979;7(2):123-36.
12. Beyer WE, Palache AM, Osterhaus AD. Comparison of serology and reactogenicity between influenza subunit vaccines and whole virus or split vaccines: A review and meta-analysis of the literature. Clin Drug Investig. 1998;15:1–12.
13. Stephenson I, Nicholson KG, Glück R, Mischler R, Newman RW, Palache AM, Verlander NQ, Warburton F, Wood JM, Zambon MC. Safety and antigenicity of

whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. Lancet. 2003;362(9400):1959-66.

14. Ehrlich HJ, Müller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, Fisher D, Berezuk G, Fritsch S, Löw-Baselli A, Vartian N, Bobrovsky R, Pavlova BG, Pöllabauer EM, Kistner O, Barrett PN; Baxter H5N1 Pandemic Influenza Vaccine Clinical Study Team. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N Engl J Med. 2008;358(24):2573-84.

15. Lin J, Zhang J, Dong X, Fang H, Chen J, et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: A phase I randomised controlled trial. Lancet. 2006;368:991–997.

16. Bungener L, Geeraedts F, Ter Veer W, Medema J, Wilschut J, Huckriede A. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine. 2008;26(19):2350-9.

17. Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA, Makarova N, Perez DR, Macdonald GH, McCullers JA. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol. 2006;13(9):981-90.

18. Hovden AO, Cox RJ, Haaheim LR. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol. 2005;62:36–44.

19. Geeraedts F, Bungener L, Pool J, Ter Veer W, Wilschut J, et al. Whole inactivated virus influenza vaccine is superior to subunit vaccine in inducing immune responses and secretion of proinflammatory cytokines by DCs. Influenza Other Respir Viruses. 2008;2:41–51.

20. http://www.emea.europa.eu/docs/enGB/document library/EPAR-Public assessment report/human/000982/WC500022673.pdf

21. http://www.baxter.com/press room/press releases/2009/10 07 09-celvapan.html

22. Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, et al. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol. 2007;179:4711–4720.

23. Geeraedts F, Goutagny N, Hornung V, Severa M, de Haan A, Pool J, Wilschut J, Fitzgerald KA, Huckriede A. Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling. PLoS Pathog. 2008;4(8):e1000138.

24. Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, Kopf M, Marsland BJ. TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. J Immunol. 2007;178(4):2182-91.

25. Kasturi SP, Skountzou I, Albrecht RA, Koutsonanos D, Hua T, Nakaya HI, Ravindran R, Stewart S, Alam M, Kwissa M, Villinger F, Murthy N, Steel J, Jacob J, Hogan RJ, García-Sastre A, Compans R, Pulendran B. Programming the magnitude

and persistence of antibody responses with innate immunity. Nature. 2011;470(7335):543-7.

26. Koyama S, Aoshi T, Tanimoto T, Kumagai Y, Kobiyama K, Tougan T, Sakurai K, Coban C, Horii T, Akira S, Ishii KJ. Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. Sci Transl Med. 2010;2(25):25ra24.

27. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A. 2004;101(15):5598-603.

28. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004;303:1529–1531

29. Mitchison NA. T-cell-B-cell cooperation. Nat Rev Immunol. 2004 Apr;4(4):308-12.

30. Wang JP, Liu P, Latz E, Golenbock DT, Finberg RW, Libraty DH. Flavivirus activation of plasmacytoid dendritic cells delineates key elements of TLR7 signaling beyond endosomal recognition. J Immunol. 2006;177(10):7114-21.

31. Norrby E, Gollmar Y. Identification of measles virus-specific hemolysis-inihibiting antibodies separate from hemagglutination-inhibiting antibodies. Infect Immun. 1975;11(2):231-9.

32. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol. 2002;3(2): 196-200.

33. Gilliet M, Boonstra A, Paturel C, Antonenko S, Xu XL, Trinchieri G, O'Garra A, Liu YJ. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. J Exp Med. 2002;195(7):953-8.

34. Stegmann T, Schoen P, Bron R, Wey J, Bartoldus I, Ortiz A, Nieva JL, Wilschut J. Evaluation of viral membrane fusion assays. Comparison of the octadecylrhodamine dequenching assay with the pyrene excimer assay. Biochemistry. 1993;32(42):11330-7.

35. Stegmann T, Morselt HW, Booy FP, van Breemen JF, Scherphof G, Wilschut J. Functional reconstitution of influenza virus envelopes. EMBO J. 1987;6:2651–9.

36. Yoshimura A, Ohnishi S. Uncoating of influenza virus in endosomes. J Virol. 1984;51(2):497-504.

37. Macdonald RA, Hosking CS, Jones CL. The measurement of relative antibody affinity by ELISA using thiocyanate elution. J Immunol Methods. 1988;106(2):191-4.

38. Bridges CB, Katz J, Levandowski R, Cox NJ. Inactivated influenza vaccines. In: Plotkin SA, Orenstein WA, Offit PA, eds. Vaccines 5th Edition: Saunders Elsevier 2008; 259-90.

39. Stanley WM. The preparation and properties of influenza virus vaccines concentrated and purified by differential centrifugation. J Exp Med. 1945;81(2): 193-218.

40. Takada A, Matsushita S, Ninomiya A, Kawaoka Y, Kida H. Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. Vaccine. 2003;21(23):3212-8.

41. Goldstein MA, Tauraso NM. Effect of formalin, beta-propiolactone, merthiolate, and ultraviolet light upon influenza virus infectivity chicken cell agglutination, hemagglutination, and antigenicity. Appl Microbiol. 1970;19(2):290-4.

42. Meng D, Hui Z, Yang J, Yuan J, Ling Y, He C. Reduced egg production in hens associated with avian influenza vaccines and formalin levels. Avian Dis. 2009;53(1): 16-20.

43. di Tommaso A, de Magistris MT, Bugnoli M, Marsili I, Rappuoli R, Abrignani S. Formaldehyde treatment of proteins can constrain presentation to T cells by limiting antigen processing. Infect Immun. 1994;62(5):1830-4.

44. Gilbert C, Svejstrup JQ. RNA immunoprecipitation for determining RNA-protein associations in vivo. Curr Protoc Mol Biol. 2006;Chapter 27:Unit 27.4.

45. Nishiya T, Kajita E, Miwa S, Defranco AL. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. J Biol Chem. 2005;280(44):37107-17.

46. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, Diaz L, Trento A, Chang HY, Mitzner W, Ravetch J, Melero JA, Irusta PM, Polack FP. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med. 2009;15(1):34-41.

47. Murphy, B. R., G. A. Prince, E. E. Walsh, H. W. Kim, R. H. Parrott, V. G. Hemming, W. J. Rodriguez, and R. M. Chanock. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J. Clin. Microbiol. 1986;24:197-202.

48. Heil F, Ahmad-Nejad P, Hemmi H, Hochrein H, Ampenberger F, Gellert T, Dietrich H, Lipford G, Takeda K, Akira S, Wagner H, Bauer S. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur J Immunol. 2003;33(11):2987-97.

49. Wang JP, Asher DR, Chan M, Kurt-Jones EA, Finberg RW. Cutting Edge: Antibody-mediated TLR7-dependent recognition of viral RNA. J Immunol. 2007;178(6): 3363-7.

50. Budimir N, Meijerhof T, Wilschut J, Huckriede A, de Haan A. The role of membrane fusion activity of a whole inactivated influenza virus vaccine in (re)activation of influenza-specific cytotoxic T lymphocytes. Vaccine. 2010;28(52):8280-7.

51. Rimmelzwaan GF, Fouchier RA, Osterhaus AD. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. Curr Opin Biotechnol. 2007;18(6):529-36.

52. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem. 1977 Dec;83(2):346-56.

53. Böttcher CSF, van Gent CM, Fries C. A rapid and sensitive sub-micro phosphorusdetermination. Anal Chim Acta. 1961;24:203–204.

Alum boosts TH2-type antibody responses to whole inactivated virus influenza vaccine in mice but does not confer superior protection

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Abstract

Clinical trials with pandemic influenza vaccine candidates have focused on aluminium hydroxide as an adjuvant to boost humoral immune responses. In this study we investigated the effect of aluminium hydroxide on the the magnitude and type of immune response induced by whole inactivated virus (WIV) vaccine. Balb/c mice were immunized once with a range of antigen doses (0.04-5 μg) of WIV produced from A/PR/8 virus, either alone or in combination with aluminium hydroxide. The hemagglutination inhibition (HI) titers of mice receiving WIV + aluminium hydroxide were 4-16 fold higher than HI titers in mice receiving the same dose of WIV alone, indicating the boosting effect of aluminium hydroxide. WIV induced a TH1 skewed humoral and cellular immune response, characterized by strong influenza-specific IgG2a responses and a high number of IFNγ-secreting T cells. In contrast, immunization with WIV adsorbed to aluminium hydroxide resulted in skewing of this response to a TH2 phenotype (high IgG1 levels and a low number of IFNγ-producing T cells).

To assess the effect of the observed immune response skewing on viral clearance from the lungs mice immunized once with 1 μg WIV without or with aluminium hydroxide were challenged with A/PR/8 virus 4 weeks later. The immunized mice showed a significant decrease in viral lung titers compared to control mice receiving buffer. However, despite higher antibody titers, mice immunized with WIV adsorbed to aluminium hydroxide suffered from more severe weight loss and had significantly higher virus loads in their lung tissue than mice receiving WIV alone. Major difference between these groups of mice was the type of immune response induced, TH2 instead of TH1, indicating that a TH1 response plays a major role in viral clearance.

Introduction

Influenza virus continues to be a major health burden. According to the World Health Organisation (WHO) the estimated number of worldwide excess deaths due to influenza is 0.25-0.5 million each year [1]. In addition to the seasonal epidemic influenza burden there is the risk of a pandemic caused by an influenza virus to which the majority of the world population is immunologically naïve. Morbidity and mortality of a pandemic influenza strain will likely be much higher than that of epidemic influenza [2]. Currently, H5N1, H7N2, H7N3, H7N7 and H9N2 have crossed the species barrier from birds to man to cause human infections on multiple occasions [3, 4, reviewed in 5]. H5N1 is considered a likely candidate for the next pandemic, having been confirmed to infect 357 people resulting in 225 deaths (WHO, Cumulative Number of Confirmed Human Cases of Avian Influenza A/ (H5N1) February 1, 2008).

The best protection against influenza virus infection remains effective vaccination [6]. Inactivated vaccines against influenza virus include whole-inactivated virus (WIV) vaccines, split virus vaccines, and subunit vaccines [7]. WIV vaccine is prepared by inactivation of influenza virus with ß-propiolactone or formaldehyde, resulting in presence of all viral proteins in their native organization without viral replication. Split influenza virus vaccine consists of chemically disrupted inactivated influenza virus. Subunit vaccines are prepared by purification of the HA and NA from inactivated and detergent-solubilized influenza virus. All of these vaccine formulations have been reported to be in clinical trials as pandemic vaccine candidates [8].

A major objective in the context of the development of pandemic vaccines is to ensure sufficient supply of vaccines despite limited production capacities for vaccine virus. Dose sparing strategies are being developed to solve this problem. One of these dose sparing strategies is the use of WIV instead of split virus or subunit since WIV induces higher antibody responses, especially at low antigen doses and in an immunologically naïve population [9, 10]. Another dose sparing strategy to increase the efficacy of pandemic influenza vaccines is addition of adjuvants to the vaccines to boost immune responses.

Adjuvants used most frequently for human vaccination are aluminium compounds, including aluminium hydroxide and aluminium phosphate [11, 12, reviewed in 13]. Prior to injection the antigen is adsorbed onto a preformed aluminium gel (Alhydrogel® or Adju-phos®). Aluminium adjuvants generally

induce a TH2 type of immune response and result in a stronger and more rapid induction of antibody titers. This makes them potentially suitable for the pandemic situation where likely only one vaccination can be achieved before the first wave of the pandemic [14]. Accordingly, in the majority of clinical trials performed to date with pandemic influenza vaccine candidates aluminium hydroxide or aluminium phosphate were used as the adjuvant [15-19, for an overview of all clinical pandemic influenza vaccine trials see 8]. Outcomes of these clinical trials are reported predominantly as hemagglutination inhibition titers and seroconversion rates. So far, six clinical studies have been reported which compare the response to non-adjuvanted and alum-adjuvanted pandemic influenza vaccines, respectively [8]. Three of these studies did not find a benefit or even a negative effect of alum adjuvants [8: Baxter study A/Vietnam/1194/2004 WIV, Sanofi Pasteur study A/Vietnam/1203/2004 split vaccine in adults (= ref 17), Novartis study A/Vietnam/1203/2004 subunit vaccine). Two studies found inconsistent results [8: GSK study H9N2 WIV, Sanofi Pasteur study A/Vietnam/1203/2004 split vaccine in the elderly]. One study reported improvement of seroprotection, seroconversion and rise of GMT by alum [8: GSK study A/Vietnam/1194/2004 WIV]

The mode of action of aluminium compounds is not completely clear yet, they may form a depot for the antigen, either at the site of injection or in the draining lymph nodes. The depot enables slow release from the site of injection and/or longer contact with cells of the immune system [13, 20]. Another mode of action could be the conversion of soluble antigen to particulate antigen facilitating phagocytosis by antigen-presenting cells [21]. Furthermore, aluminium compounds induce priming of B cells and accumulation of IL-4 producing Gr1+ myeloid cells facilitating B cell responses [22].

In this study we aim to determine if aluminium hydroxide is indeed a suitable adjuvant for influenza vaccines, using two vaccine formulations, WIV vaccine and subunit vaccine, in a mouse model system. H5N1 vaccines are known to be poorly immunogenic, inducing low antibody titers in both experimental animals and man [23-25]. To mimic this, H1N1 A/Puerto Rico/8/34 (PR8) virus was used as a model influenza virus. PR8 has a low immunogenicity compared to the commonly used H3N2 strains (L. Bungener, unpublished observation). Moreover, PR8 causes symptomatic infection in mice and can therefore be used in a challenge model to test the protective efficacy of the vaccines. We determined the effect of addition of aluminium hydroxide to PR8 influenza vaccines on antibody titers, phenotype of

immune response and protective capacity of the vaccines upon challenge. Aluminium hydroxide boosted the antibody response but altered the phenotype of the immune response. Moreover, despite 4-16 times higher hemagglutination inhibition titers in mice receiving aluminium hydroxide–adjuvanted WIV clearance of influenza virus from the lungs was decreased.

Materials and methods

VIRUS, SUBUNIT AND VIROSOMES

PR8 influenza virus (H1N1 subtype) grown on Madin-Darby canine kidney cells (MDCK) was kindly provided by Solvay Pharmaceuticals (Weesp, The Netherlands). The virus was purified, inactivated by treatment with formaldehyde and used as whole inactivated virus (WIV). For inactivation, virus was incubated twice with a freshly prepared 4% formaldehyde/10% sucrose solution in buffer containing 5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA (HNE buffer) at a final concentration of 0.02% formaldehyde. Inactivation was carried out for 24 hr at 4°C under continuous stirring, maintaining a neutral pH during the entire incubation. After inactivation, the virus was dialysed against HNE buffer. Virus inactivation was confirmed by standard titration of the virus preparation on MDCK cells.

Subunit material was prepared by solubilizing inactivated virus for 3 hours under continous rotation in 0.3 mg/ml Tween-80, 1.5 mg/ml hexadecyltrimethylammonium bromide (CTAB) in phosphate-buffered saline (PBS). The viral nucleocapsid was removed from the preparation by ultracentrifugation. Detergents were removed by overnight absorption onto Biobeads SM2 (Bio-Rad, Hercules, CA). Finally, the subunit material was filtered through a 0.45 μm filter.

Virosomes for stimulation during the ELISPOT assay were prepared essentially as described previously [26-29]. In short, formaldehyde-inactivated virus (1.5 μmol of viral membrane phospholipids) was solubilized in 100 mM octa(ethyleneglycol)-n-dodecyl monoether ($C_{12}E_8$), (Calbiochem, San Diego, CA) in HNE buffer. The nucleocapsid was removed from the preparation by ultracentrifugation. Subsequently, the detergent $C_{12}E_8$ was extracted from the supernatant with BioBeads SM2 resulting in the formation of virosomes. Virosomes were purified on a discontinuous sucrose density gradient. Finally, the virosomes were dialyzed against HNE buffer and filtered through a 0.45 μm filter. Viral, virosomal and subunit protein content was determined according to Lowry

[30]. HA content was assumed to be one third of total viral protein for WIV (based on the constitution of influenza virus particles) and to be equal to total protein for virosomes and subunit (based on silver-stained SDS polyacrylamide gels run under reducing and non-reducing conditions). Equal HA amounts in the vaccine preparations was verified by SDS PAGE.

IMMUNIZATION OF MICE

Specified-pathogen-free female Balb/c mice were purchased from Harlan CPB (Zeist, The Netherlands) and used at 8 to 10 weeks of age. The protocol for the animal experiments described in this paper was approved by the Animal Experimentation Ethical Committee of the University of Groningen.

The amount of HA protein in the vaccines was 5 μg, 1 μg, 0.2 μg or 0.04 μg. Alhydrogel (Gerbu Biotechnik, Gaiberg, Germany; 2% suspension) was added to the vaccines at a final concentration of 40% v/v and the mixture was gently rotated for 6 hours at 4°C just before vaccination of the mice. Vaccines without Alhydrogel adjuvant were incubated in the rotator simultaneously. All adjuvanted vaccine formulations contained 200 μg of aluminium per vaccination dose. Mice (6-8 animals per group) were immunized once intramuscularly (i.m.) with whole inactivated virus (WIV) or subunit vaccine with or without aluminium hydroxide in 50 μl per mouse divided over two hind legs. Four weeks after immunization, mice were bled under anaesthesia, sacrificed and spleens were harvested. Spleen cells were isolated and used in ELISPOT assays. Serum was collected for antibody assays.

ELISPOT ASSAY

ELISPOT analyses were performed according to a protocol adapted from the method described by Miyahira [31]. ELISA plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with purified anti-mouse IFNγ or IL-4 (rat IgG1, Pharmingen, San Diego, CA) overnight at 37°C. Plates were washed three times with sterile PBS Tween (PBS + 0.02% Tween 20) and incubated with blocking buffer (PBS containing 4% RIA Grade BSA) for 1 hr. Spleen cells were plated in different quantities in medium containing 5% FCS and incubated overnight with or without 1 μg per well of virosomal protein. Virosomes were chosen for restimulation since they stimulate only HA- and NA-specific T cells (which were to be studied here). In previous studies virosomes were found to be superior to subunit material in T cell stimulation and the optimal stimulation dose was

determined to be 1 μg (A. Huckriede, unpublished observations). Subsequently, cells were lysed by 10-min incubation in water and plates were washed five times with PBS Tween. IFNγ was detected using biotinylated anti-mouse IFNγ antibody and streptavidin-alkaline phosphatase (Pharmingen). The substrate for the alkaline phosphatase was 1 mg/ml 5-bromo-4-chloro-3-indolylphosphate in water containing 6 mg/ml agarose (Sigma), 9.2 mg/ml 2-amino-2-methyl-1-propanol (Sigma) and 0.08 μl/ml Triton X-405. Spots were developed for 3 h at 37°C and counted using an automated ELISA-spot assay video analysis system (A EL VIS, Hannover, Germany). Background (spleen cells incubated without virosomal protein) was usually less than 5 spots per 10^6 cells plated. This background was subtracted from the number of spots observed in wells containing spleen cells incubated with antigen to obtain the number of IFNγ-secreting cells.

Hemagglutination inhibition (HI) assay

For determination of HI titers in serum, 75 μl of serum was first inactivated by incubation for 30 min at 56°C. In order to reduce non-specific hemagglutination, 225 μl of a 25% kaolin suspension was added. The mixture was vortexed and incubated for 20 min at room temperature. After centrifugation for 2 min at 6500× *g*, 50 μl of the supernatant was transferred in duplicate to 96-well round-bottom plates (Greiner) and serially diluted twofold in PBS. Next, 4 hemagglutination units (HAU) of influenza PR8 virus were added to each well in a volume of 50 μl. The content of each well was gently mixed with a multichannel pipette and plates were incubated for 40 min at room temperature. Finally, 50 μl of a 1% guinea pig erythrocyte suspension in PBS was added to each well and hemagglutination was allowed to proceed for 3 h at room temperature. The highest serum dilution capable of preventing hemagglutination was scored as the HI titer. Detection limit of this assay was 4 and non-responding sera were assigned an arbitrary titer of half the detection limit (HI titer of 2).

IgG, IgG1 and IgG2a ELISAs

Influenza HA-specific antibody responses were determined using ELISA, as previously described [32]. ELISA plates (Greiner, Alphen a/d Rijn, Netherlands) were coated with 0.2 μg of influenza PR8 subunit antigen per well. Appropriate dilutions of sera of individual mice were applied to the plates, serially diluted twofold in PBS/Tween (PBS containing 0.05% Tween20), and then incubated for 1.5 h at 37°C. Subsequently, plates were washed and incubated with horseradish

peroxidase-conjugated goat antibodies directed against mouse IgG, IgG1 or IgG2a (all 1:5000, Southern Biotechnologies). After incubation of the plates with mouse Ig isotype- and subtype-specific conjugates for 1 h at 37°C, plates were washed twice with PBS/Tween, and once with PBS. Antibodies were detected using substrate buffer (50 mM phosphate buffer pH 5.5 containing 0.02% *o*-phenylenediamine and 0.006% H_2O_2). Plates were developed in the dark for 30 min at room temperature after which the reaction was stopped by addition of 50 µl 2 M H_2SO_4 per well, and absorbances were read at 492 nm (A_{492}) using a SPECTRA I ELISA reader (SLT, Salzburg, Austria). Titers are given as the log10 or geometric mean titer of the reciprocal of the sample dilution calculated to correspond to an A_{492} of 0.2.

For IgG1 and IgG2a, a calibration curve was obtained by coating ELISA plates overnight with 0.1 µg/ well of goat anti mouse IgG (Southern Biotechnologies) instead of subunit antigen and incubating with increasing concentrations of purified mouse IgG1 or IgG2a (Southern Biotechnologies) instead of serum dilutions. Results for IgG1 and IgG2a are expressed as concentrations of influenza HA-specific IgG1 and IgG2a.

CHALLENGE EXPERIMENTS

For challenge experiments mice were vaccinated i.m. with 1 µg of HA in WIV or subunit vaccine, adsorbed or not adsorbed to aluminium hydroxide. Control mice were injected with buffer only. Four weeks after vaccination mice were challenged intranasally under anaesthesia with $1x10^4$ $TCID_{50}$ PR8 influenza virus in 40 µl of PBS [33]. The dose of challenge virus was chosen to allow the detection of possible differences in the protective capacities of the tested vaccine formulations.
Mice were monitored twice a day for signs of clinical illness by weighing the animals and observing their appearance and activity. After three days mice were bled and sacrificed. The left lung lobes were collected and stored in 1 ml of PBS before homogenisation. One of the right lung lobes was isolated and frozen in liquid nitrogen for histopathology. Another right lobe was inflated with formalin for fixation and embedded in paraffin.

DETERMINATION OF VIRUS TITERS IN LUNGS OF CHALLENGED MICE

Virus titers in the lung tissue were determined by homogenising lung tissue, pelleting debris by centrifugation and collecting the supernatant containing the virus. This lung homogenate was stored at -80°C until virus titration. Virus titration was performed on MDCK cells seeded in 96 well plates. Two-fold

dilutions (in duplicate) of virus-containing lung homogenate supernatant were prepared in a flat-bottom 96 well plate. Medium was removed from the monolayer of MDCK cells and 50 μl dilutions of lung homogenates were transferred to the cells. After 1 hour 50 μl medium containing 7.5 μg TPCK trypsin (Sigma) was added per well followed by a 72 hour incubation at 37°C, 5% CO_2. Supernatants were collected and put in a roundbottom 96 well plate. Addition of 50 μl of a 1% solution of guinea pig erythrocytes and incubation for 2 hours enabled readout of the dilution at which virus was still present (indicated with hemagglutination), which represents the virus titer in the lungs. Next, the ^{10}log virus titer was calculated per gram of lung tissue.

STATISTICAL ANALYSIS

The unpaired Student's *t*-test was used to determine if the difference in immune responses observed between groups of mice was significant. HI and IgG titers were log-transformed prior to statistical analysis. P values are given and in general a p value of $p < 0.05$ was considered significant.

Results

EFFECT OF ALUMINIUM HYDROXIDE ON THE MAGNITUDE OF THE ANTIBODY RESPONSE TO WIV

In order to elucidate potential dose-sparing effects of aluminium hydroxide on antibody responses to WIV antigen, mice were immunized once with decreasing doses of WIV PR8. The highest immunization dose was 5 μg and the other experimental groups were injected with five-fold lower doses (1 μg, 0.2 μg and 0.04 μg). The decision to immunize once was based on the assumption that in case of a pandemic it will most probably not be possible to immunize and boost before the first wave of the pandemic strikes [34]. Only the highest amount of non-adjuvanted WIV (5 μg of HA) was sufficient to induce hemagglutination inhibition (HI) titers > 40 in 5 out of 8 mice with a geometric mean (GM) HI titer of 37 (Figure 1A). In humans, an HI titer of 40 is considered to be protective in at least 50% of the population [35, 36]. Lowering the antigen dose five-fold to 1 μg resulted in a three-fold decrease of the GM HI titer to 13 with only one animal exhibiting a HI titer >40. Immunization with 0.2 μg and 0.04 μg HA without aluminium hydroxide resulted in GM titers of 5 and 4, in these groups half of the mice had no detectable

HI titer and none of the animals showed a titer > 40. These comparatively low HI titers indicate that the PR8 influenza virus is indeed poorly immunogenic in a single dose regimen without adjuvant.
Addition of Alhydrogel to the WIV vaccine strongly and significantly enhanced HI titers to the extent that even immunization with 0.2 μg HA resulted in HI titers > 40 in 7 out of 8 mice (Figure 1A). GM HI titers were 175 for 5 μg HA, 147 (1 μg), 83 (0.2 μg) and 16 (0.04 μg), respectively. P values for differences between vaccination with and without alum were 0.003, 0.0006, 0.00003, and 0.02 for the indicated antigen doses, respectively. Thus, upon addition of aluminium hydroxide to WIV, HI titers in mice were boosted by 4-16 fold depending on antigen dose.

In contrast to this clear boost of HI titers, total influenza-specific IgG levels were only marginally higher at all antigen concentrations upon addition of aluminium hydroxide to WIV (Figure 1B). This rise in specific total IgG was only significant for the lower antigen dose of 0.2 μg indicating that there was no clear correlation between HI titer and total specific IgG in this experiment.

Effect of aluminium hydroxide on the phenotype of the immune response to WIV

In order to clarify the incongruence observed between HI titers and total IgG and to obtain insight into the phenotype of the antibody response we determined HA-specific IgG1 and IgG2a levels in sera of immunized mice. IgG1 was used as a measure for a TH2-biased antibody response and IgG2a as a measure of a TH1-biased antibody response.

WIV immunization induced low HA-specific IgG1 levels and high specific IgG2a responses (Figure 2). Addition of Alhydrogel to WIV strongly altered the phenotype of the immune response. For all vaccine doses tested, influenza-specific IgG1 responses increased significantly while IgG2a responses decreased significantly compared to responses in animals that received WIV vaccine alone ($p \leq 0.005$ in all cases). All of the animals immunized with WIV alone had higher influenza-specific IgG2a responses than IgG1 responses, resulting in IgG2a/IgG1 ratios > 1 (Table 1). In contrast, IgG2a/IgG1 ratios in animals immunized with WIV adsorbed to aluminium hydroxide decreased to 0.06-0.4 indicating a TH2-biased antibody response. When IgG1 and IgG2a amounts are added the sum shows the same vaccine dose dependent decrease as observed for total IgG titers (Figure 1B). Upon addition of aluminium hydroxide the increase in IgG1 is almost

compensated by a decrease in IgG2a for all vaccine doses. This explains why the titers of total IgG do not increase much upon addition of alum. Moreover, it implies that HI titers are more strongly correlated with IgG1 than with IgG2a.

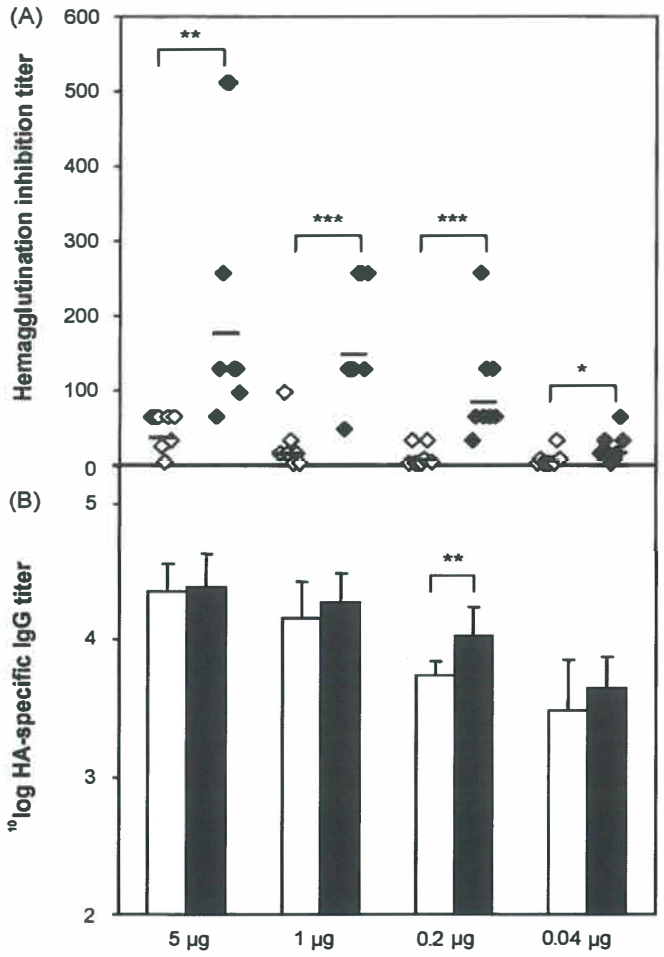

Figure 1. *Hemagglutination inhibition titers (A) and total IgG responses (B) four weeks after a single i.m. immunization with decreasing doses (5 µg, 1 µg, 0.2 µg and 0.04 µg) of WIV vaccine without (open symbols/white bars) and with (closed symbols/black bars) aluminium hydroxide. Results for HI are depicted as responses of individual mice and geometric mean HI titers are given (black lines). For IgG responses the average ^{10}log IgG titers ± standard deviation are given (n=8 mice per group). Titers of influenza HA-specific IgG were determined in sera by ELISA using subunit antigen as the coating substance. Levels of significance are depicted as follows: * p<0.05, ** p<0.01 and *** p<0.001.*

Table 1. IgG2a/IgG1 ratio after one immunization with WIV vaccine with or without aluminium hydroxide

Vaccine	5 µg HA	1 µg HA	0.2 µg HA	0.04 µg HA
WIV	35.4	50.3	252.8	26.9
WIV + Alhydrogel	0.09	0.17	0.06	0.40

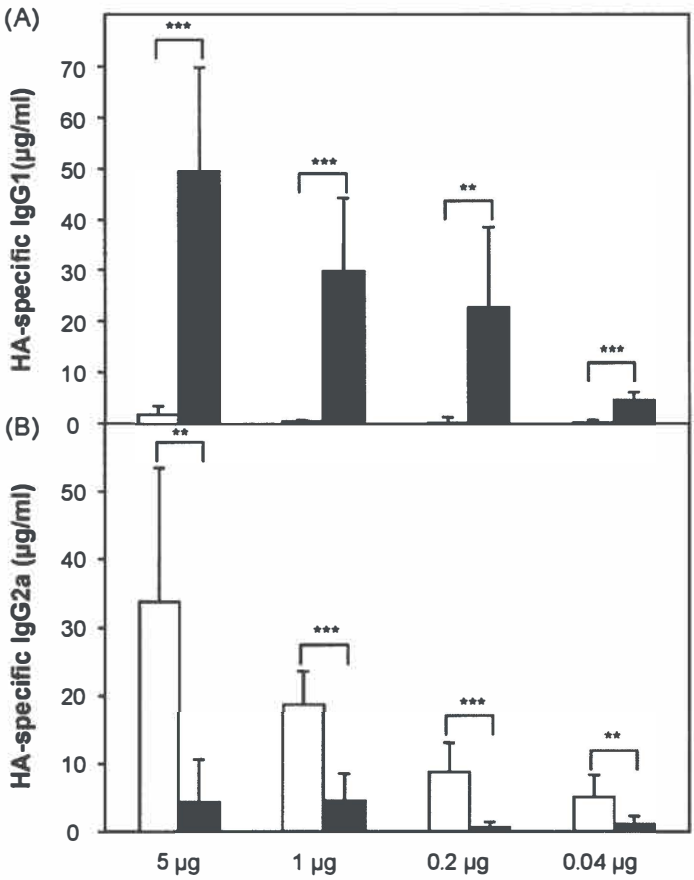

Figure 2. IgG1 (A) and IgG2a (B) antibody responses in mice immunized i.m. with 5 µg, 1 µg, 0.2 µg and 0.04 µg of WIV vaccine without (white bars) and with (black bars) aluminium hydroxide. Quantities of influenza HA-specific IgG1 and IgG2a four weeks after immunization were determined in sera by ELISA using subunit antigen as the coating substance and a calibration curve of murine IgG1 or IgG2a on plates coated with goat anti mouse IgG. Average quantities of influenza-specific IgG1 and IgG2a ± standard deviation are depicted (n=8 mice per group).

To determine if the strong bias towards a TH2 immune response observed upon addition of the aluminium hydroxide to WIV extends to the T helper cell response, spleen cells of immunized mice were harvested and tested in an ELISPOT assay. Influenza antigen-specific IFNγ responses were significantly ($p = 0.0008$) higher in mice immunized with WIV alone compared to mice immunized with WIV + alum (Figure 3). The IL-4 response in spleen cells on the other hand was significantly higher when mice were immunized with WIV combined with Alhydrogel ($p = 0.02$). Mice immunized with WIV alone had a TH1/TH2 ratio > 1 with an average of 3.3 whereas animals immunized with WIV adsorbed to aluminium hydroxide had an average ratio of 0.8. Thus, immunization with WIV alone results in a dominant IFNγ response whereas IL-4 production is dominant in spleens from animals immunized with WIV adsorbed to aluminium hydroxide.

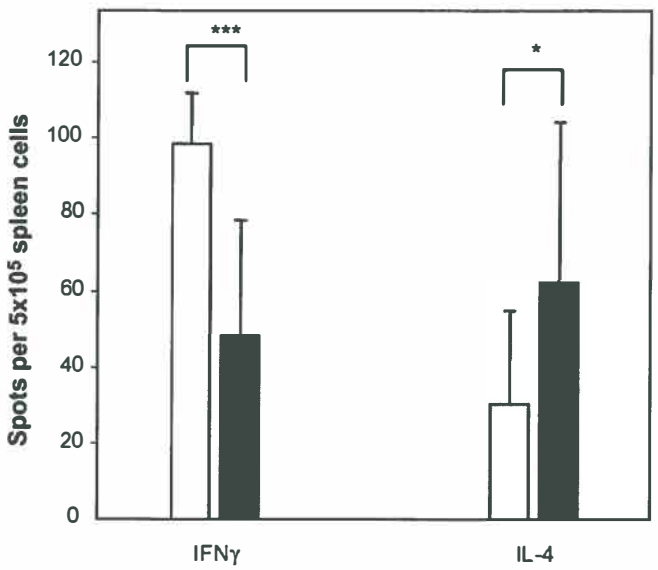

Figure 3. *Cytokine-producing T cells after immunization with 5 μg WIV vaccine without (white bars) and with (black bars) aluminium hydroxide. Spleen cells were isolated 4 weeks after immunization and stimulated overnight with virosomes (1 μg of protein per well). IFNγ- and IL-4-producing T cells were detected by ELISPOT.*

Effect of the phenotype of the immune response on viral clearance from the lungs

To assess the effect of aluminium hydroxide addition to influenza vaccines on virus clearance, immunized mice were challenged with a high dose of PR8. For

this purpose, mice were immunized with 1 μg HA formulated as WIV or subunit vaccine, with or without previous absorption to aluminium hydroxide. Four weeks after immunization of the mice, serum samples were taken and animals were infected with $1x10^4$ $TCID_{50}$ of PR8 virus.

Subunit antigen was added as a vaccine candidate in the challenge studies to further clarify the role of the type of immune response (TH1 versus TH2) on the ability to respond effectively to a virus challenge. Protein antigens alone typically induce a TH2 type of immune response characterized by high IgG1 and low IgG2a. Since aluminium hydroxide also stimulates a TH2 response, this vaccine-adjuvant combination might work synergistically.

All mice showed strong antibody responses four weeks after immunization, at the time of virus challenge (Table 2). As expected, the results for subunit-immunized mice corroborate the TH2 bias of this protein antigen. Antibody profiles of subunit-immunized mice prior to challenge were TH2 dominated with moderate IgG1 induction and almost no IgG2a. Addition of aluminium hydroxide to subunit vaccine significantly boosted IgG and IgG1 antibody responses ($p = 0.003$ and $p = 0.002$). Pre-challenge antibody titers for WIV with and without aluminium hydroxide were similar to titers observed in previous immunization experiments while the amount of IgG1 in the WIV/alum group was higher for reasons unknown. As expected, significant increases in IgG1 ($p = 0.001$) and decreases in IgG2a ($p = 0.01$) were measured upon addition of aluminium hydroxide to WIV.

Table 2. Prechallenge influenza HA-specific antibody responses to 1 μg WIV or subunit vaccine with or without aluminium hydroxide

Vaccine	Total influenza HA-specific IgG (^{10}log ± stdev)	IgG1 (μg/ml ±stdev)	IgG2a (μg/ml ±stdev)	IgG2a/IgG1
WIV	4.02±0.23	3.1±1.4	31.4±23.1	11.9
WIV + Alhydrogel	4.16±0.21	130.5±68.5	2.9±2.5	0.04
Subunit	2.61±0.42	3.3±3.1	0.1±0.1	ND
Subunit + Alhydrogel	3.79±0.28	41.7±22.6	2.4±3.3	0.06

ND = not detectable because 3/6 mice had no specific IgG2a

After virus challenge, control mice showed signs of illness (increased breathing rate, ruffled fur and reduced activity) and substantial weight loss indicating that the challenge was severe (Figure 4A). Control animals injected with buffer lost 11.4% of their weight in the three days following challenge and all of the animals in this group were still losing weight at the moment of sacrifice. Mice immunized with subunit and subunit + Alhydrogel lost 12.6% and 7.5% of their original weights respectively. These weightlosses were not significantly different from those in the control group ($p = 0.6$ and $p = 0.1$, respectively). Like the animals in the control group, these mice were still losing weight and showing signs of illness at the time of sacrifice. Mice immunized with WIV or WIV + aluminium hydroxide showed a weight gain during the first one and a half days after challenge, followed by weight loss in the next day and a stabilisation in the last hours before termination. Animals immunized with WIV + Alhydrogel had an average weight loss of 5.3% three days after challenge, this weight loss was lower than that in the control group ($p = 0.05$). In contrast to all other experimental groups, WIV-immunized animals had an average weight gain on day 3 of 1.5% and this group was statistically different from the control group ($p = 0.0004$). Moreover, WIV-immunized mice did not show signs of illness and remained active.

On day three, animals were sacrificed and virus titers in the lungs were determined (Figure 4B). Buffer-injected mice had an geomean titer of $10^{8.8}$. With GM titers of $10^{8.4}$ (subunit alone) and $10^{8.1}$ (subunit + aluminium hydroxide), virus titers in lungs of subunit-immunized mice were not statistically different from those in the control group ($p = 0.14$ and $p= 0.4$, respectively). In contrast, the GM virus titer in mice immunized with WIV ($10^{5.5}$) was about 3 logs lower than that in the control group and this difference was statistically highly significant ($p = 0.005$). WIV + aluminium hydroxide immunized mice also showed significantly lower virus titers than the control group with a GM lung titer of $10^{7.7}$ ($p = 0.0006$). Yet, the virus titer in these mice was significantly higher ($p = 0.04$) than the titer in mice immunized with WIV alone.despite of high amounts of influenza-specific IgG1 and thus presumably a much higher HI titer (in the experiment depicted in Fig.1 the difference in HI titer after vaccination with 1 μg WIV with or without alum was factor 12) (Figure 1A).

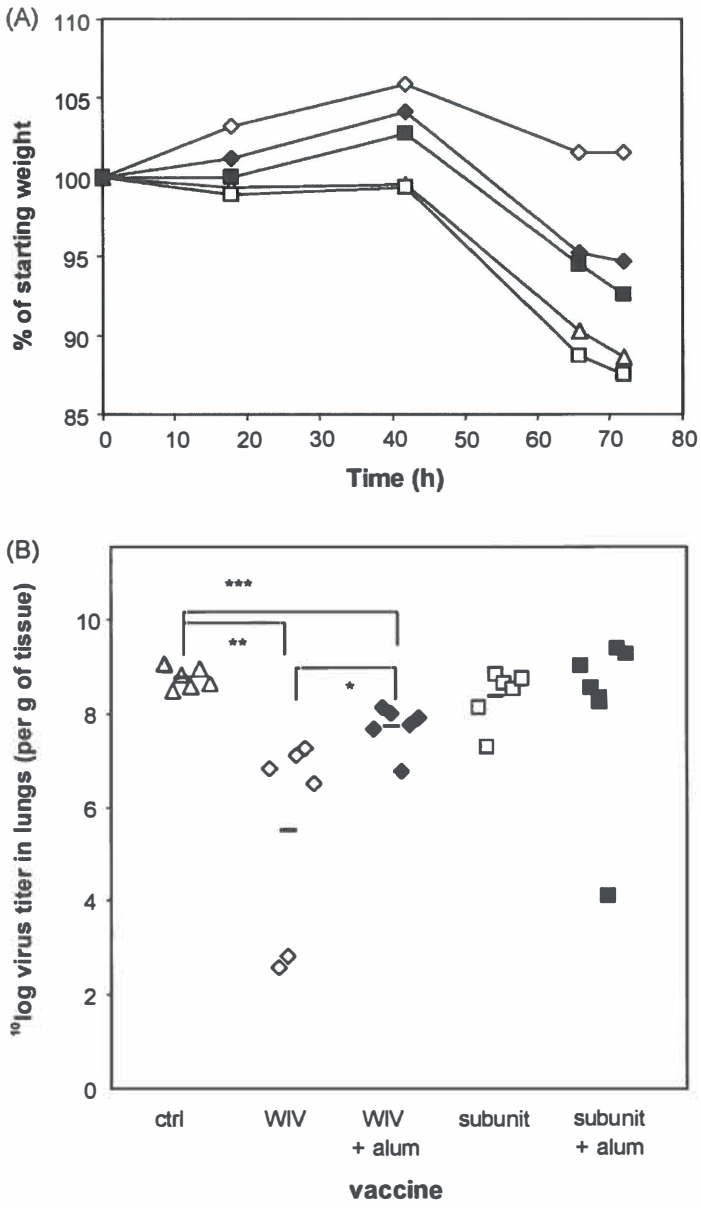

Figure 4. Body weight (A) and virus titers in lungs (B) of mice immunized with WIV or subunit with and without aluminium hydroxide and challenged intranasally 4 weeks later with PR8 virus. Animals were vaccinated i.m. with WIV (diamonds) or subunit (squares) containing 1 μg of HA. As a control mice were injected with HNE buffer (triangles). Vaccines were given without aluminium hydroxide (open symbols) or adjuvanted with aluminium hydroxide (closed symbols). Mice were challenged with 1x10^4 TCID$_{50}$ of live PR8 virus given under isoflurane anesthesia to ensure inhalation of the virus into the lungs. Three days after the virus challenge the animals were sacrificed and lungs were harvested. Average body weight per group (n=6 per group) is depicted in (A), virus titers are expressed as the ^{10}log virus titer per gram of lung tissue of individual mice (B).

Discussion

In this study, the influence of aluminium hydroxide on the magnitude, the phenotype and the protective capacity of the immune response induced by WIV influenza vaccine was determined. WIV was chosen because it is one of the promising pandemic influenza vaccine candidates currently investigated in clinical trials [9, 10]. Aluminium hydroxide was chosen because it is used as an adjuvant in most of the clinical trials currently performed in the context of the development of a pandemic influenza vaccine and because it is one of the few adjuvants licensed in humans [8]. Aluminium hydroxide, a TH2 type adjuvant, boosts antibody responses and protection against influenza virus infection is mediated primarily by influenza-specific antibodies [11, 13, 37].

Data presented in this paper show that absorption of WIV to aluminium hydroxide strongly stimulates HI titers and IgG1 titers upon vaccination of mice but suppresses IgG2a titers and impairs the induction of IFNγ-producing TH1 cells. Therefore, addition of aluminium hydroxide to WIV increases the magnitude of the antibody response (HI and IgG1) but changes the phenotype dramatically from a TH1-dominated to a TH2-dominated response. Most importantly, despite the higher HI titers induced by WIV + aluminium hydroxide, addition of the adjuvant to the vaccine had no beneficial effect on virus clearance from the lungs upon virus challenge. Total virus titers in lungs of mice immunized with WIV in combination with aluminium hydroxide were even significantly higher than virus titers in lungs of animals immunized with WIV alone.

The limited protective capacity of a TH2 response was confirmed in mice immunized with subunit vaccine. This influenza vaccine, consisting only of influenza protein, is a TH2 type vaccine inducing primarily IgG1 and IL-4 responses [38]. Addition of the TH2 adjuvant aluminium hydroxide did not change the phenotype of the immune reaction evoked by the vaccine but simply boosted the antibody response (total IgG, IgG1 in particular) significantly. However, this TH2 type of immune response did not confer any protection against virus replication in the lungs of immunized mice. Thus, even though total specific IgG levels are similar in mice immunized with WIV alone and animals immunized with subunit + aluminium hydroxide, protection against virus replication in lungs is only observed in WIV immunized mice exhibiting a TH1 skewed immune response. This strengthens the hypothesis that a TH2 type of immune response is not optimal for protection against virus replication. Again, high HI titers alone do

not necessarily correlate with protection against challenge with high virus load, at least not in the mouse model. The use of the HI titer as single correlate of protection can thus be misleading.

The effect of aluminium hydroxide adjuvant on the antibody response and protective capacity of whole inactivated virus vaccine has recently been studied in mice for two vaccines derived from recombinant H5N1 strains [39, 40]. In the first study, HI titers for the adjuvanted versus the non-adjuvanted groups were statistically not different after two immunizations except for the 0.02 μg group immunized with A/HK/213/04-derived vaccine which developed a low HI titer of about 11 with adjuvant but no titer without adjuvant [39]. Protection from death or weight loss did not correlate with HI titers and occurred even in the absence of any hemagglutination inhibiting antibodies. Addition of aluminium hydroxide to the vaccine increased survival rates especially upon heterologous challenge. In contrast, in the second study survival rates at low antigen doses were found to be higher for non-adjuvanted whole inactivated virus vaccine than for alum-adjuvanted vaccine [40]. This was true for outbred CD1 mice whereas in Balb/c mice protection after immunization with and without alum did not differ, although the latter results are not shown. Unfortunately, the phenotype of the immune response in terms of IgG1 vs IgG2a and IFNγ- vs IL-4-producing T cells was not investigated in either of these studies and correlates of protection therefore remain unclear. The effect of the site of immunization and the immunization scheme on the magnitude and phenotype of the elicited immune response to the adjuvanted and non-adjuvanted vaccines certainly deserves further investigation.

Immune responses to a vaccine are determined by both the composition and formulation of the vaccine and the presence and nature of added adjuvants. We and others have previously shown that WIV induces a stronger and more TH1-skewed antibody response than split, subunit or virosomal vaccine formulations at least after a single immunization [38, 41, 42]. Together with the data presented here and unpublished results of our group on H5N1 vaccines these studies clearly demonstrate that the relative magnitude of the immune response and the TH profile induced are independent of vaccine strain and vaccine dose but rather depend on vaccine formulation and possibly on the number of immunizations given [42].

In general, induction of influenza-specific antibodies by vaccination clearly correlates with protection against infection [43, 44], but the subtype of antibody

mediating protection is less clear. In the mouse, the antibody profile induced by influenza virus infection is predominantly of the TH1 type, characterized by high IgG2a levels and low IgG1 levels and earlier reports imply that virus-specific IgG2a is important for protection [45-48]. Superiority of a TH1 skewed immune response or a mixed TH1/TH2 response over a TH2 type response for protection from influenza virus infection as shown here has also been demonstrated by Hovden et al and recently by Huber et al [42, 49]. In the Hovden study, mice were immunized once with A/Panama/2007/99 (H3N2) WIV or split vaccine resulting in strongly IgG2a-dominated and IgG1-dominated responses, respectively [41]. Upon challenge of the immunized mice with a non-lethal dose of virus, a self-limiting upper respiratory tract infection occurred. After one dose of vaccine, WIV-immunized mice had in general lower virus titers in nose wash samples than control animals or animals immunized with split virus vaccine which would confirm that the WIV-induced TH1 response is superior [42]. Yet, after 2 immunizations virus shedding was lowest in mice immunized with split vaccine. This might indicate that a mixed TH1/TH2 response can be effective in controlling virus growth provided that sufficient amounts of IgG2a are present or that cellular immune mechanisms coming up after a second vaccination also play a role in protection. However, virus titers in the Hovden study were low and variation within experimental groups was extensive. Strong conclusions can therefore not be drawn. In the Huber study, mice were vaccinated with an HA DNA vaccine, with a viral replicon system encoding HA or with a combination of both to induce IgG1-dominated, IgG2a-dominated and mixed IgG1/IgG2a responses, respectively [49]. IgG2a alone protected mice as efficiently from lethal challenge as a mixture of IgG1 and IgG2a. In contrast, IgG1 alone only protected from mild virus challenge but provided insufficient protection upon high dose challenge. As possible reasons for the superiority of IgG2a the affinity of this subtype for complement factors and Fc receptors is discussed which would both contribute to efficient clearance of virus by phagocytosis and antibody-mediated cellular cytotoxicity [49]. In contrast, IgG1 does not lead to complement activation and has only weak affinity for Fc receptors.

In humans, HI titers are so far the only correlate of protection measured for the evaluation of seasonal influenza vaccines as well as pre-pandemic vaccine candidates. There are a number of studies, primarily in healthy young adults, indicating that the median HI titer protecting 50% of vaccinated individuals against influenza virus is 28 [reviewed in 36]. Antibody subtypes, in particular the

levels of IgG1, the human equivalent of murine IgG2a, are not measured in clinical trials. Consequently, the roles of different antibody subtypes for protection against influenza virus infection in humans are so far unclear. Early studies indicate, however, that also in the human situation infection induces predominantly the TH1 antibody subtype IgG1 and provides the best protection against re-infection by homotypic and heterotypic virus strains [50, 51]. Moreover, a decline in the TH1 response in elderly is correlated with reduced influenza vaccine efficacy, adding further evidence to the importance of the TH1 response for protection against infection [52]. It is thus likely that similar to the situation in mice antibody subclass distribution is important for the protective effect of influenza vaccination also in humans and more information on this issue is urgently needed.

In summary, we show in this study that addition of aluminium hydroxide to WIV vaccines is not beneficial for prevention of virus replication in the lungs and suggest that this is caused by the switch in immune response from TH1 to TH2 caused by this adjuvant. The fact that mice immunized with WIV alone perform better upon challenge with live virus than mice immunized with WIV adjuvanted with aluminium hydroxide, despite the boost of the HI and IgG1 titers, is a clear indication that the phenotype of the immune response is of crucial importance for protection. Further research into pandemic influenza vaccine should therefore take into account that the use of HI titers or total IgG titers alone as readout for vaccine efficacy may not allow reliable prediction of the protective capacity of an influenza vaccine. We therefore strongly recommend to monitor also qualitative parameters of the immune response during pandemic vaccine trials to obtain more insight into the best correlates of protection for influenza vaccines.

Acknowledgements

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References

1. Molinari NA, Ortega-Sanchez IR, Messonnier ML, Thompson WW, Wortley PM, Weintraub E et al. The annual impact of seasonal influenza in the US: measuring disease burden and costs. Vaccine. 2007;25(27):5086-96.
2. Murray CJ, Lopez AD, Chin B, Feehan D, Hill KH. Estimation of potential global pandemic influenza mortality on the basis of vital registry data from the 1918-20 pandemic: a quantitative analysis. Lancet. 2006;368(9554):2211-8
3. Peiris M, Yuen KY, Lueng CW, Chan KH, Ip PLS, Lai RWM et al. Human infection with influenza H9N2. Lancet 1999; 354(9182):916-917.
4. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V et al. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci. 2004;101(5):1356-61.
5. Wong SS, Yuen KY. Avian influenza virus infections in humans. Chest. 2006;129(1):156-68.
6. Nichol KL, Treanor JJ. Vaccines for seasonal and pandemic influenza. J Infect Dis. 2006;194 Suppl 2:S111-8.
7. Stephenson I, Nicholson KG. Influenza: vaccination and treatment. Eur Respir J. 2001;17(6): 1282-93.
8. www.who.int/vaccine research/diseases/influenza/flu trials tables/en/index3.html
9. Nicholson KG, Tyrrell DA, Harrison P, Potter CW, Jennings R, Clark A et al. Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. J Biol Stand. 1979;7(2):123-36.
10. Stephenson I, Nicholson KG, Gluck R, Mischler R, Newman RW, Palache AM et al. Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. Lancet. 2003;362(9400):1959-66.
11. Cox JC, Coulter AR. Adjuvants--a classification and review of their modes of action. Vaccine. 1997;15(3):248-56.
12. Guy B. The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol. 2007;5(7): 505-17.
13. Gupta RK. Aluminium compounds as vaccine adjuvants. Adv Drug Deliv Rev. 1998;32(3): 155-172.
14. Hall IM, Gani R, Hughes HE, Leach S. Real-time epidemic forecasting for pandemic influenza. Epidemiol Infect. 2007;135(3):372-85
15. Hehme N, Engelmann H, Kunzel W, Neumeier E, Sanger R. Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. Med Microbiol Immunol (Berl). 2002;191(3-4):203-8.
16. Hehme N, Engelmann H, Kuenzel W, Neumeier E, Saenger R. Immunogenicity of a monovalent, aluminum-adjuvanted influenza whole virus vaccine for pandemic use. Virus Res. 2004;103(1-2):163-71.
17. Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J et al. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. Lancet 2006;367(9523):1657-64.

18. Lin J, Zhang J, Dong X, Fang H, Chen J, Su N et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet 2006;368(9540):991-97.

19. Vajo Z, Kosa L,Visontay I, Jankovics M, Jankovics I. Inactivated whole virus influenza A (H5N1) vaccine. Emerg Inf Dis 2007;13:807-8.

20. Romero Méndez IZ, Shi Y, HogenEsch H, Hem SL. Potentiation of the immune response to non-adsorbed antigens by aluminum-containing adjuvants. Vaccine. 2007;25(5):825-33

21. Mannhalter JW, Neychev HO, Zlabinger GJ, Ahmad R, Eibl MM. Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation.
Clin Exp Immunol. 1985;61(1):143-51.

22. Jordan MB, Mills DM, Kappler J, Marrack P, Cambier JC. Promotion of B cell immune responses via an alum-induced myeloid cell population. Science. 2004;304(5678):1808-10.

23. Stephenson I, Bugarini R, Nicholson KG, Podda A, Wood JM, Zambon MC, Katz JM. Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. J Infect Dis. 2005;191(8):1210-5.

24. Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E et al. Safety and antigenicity of non-adjuvanted and MF-59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 2001; 357(9272):1937–43.

25. Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O'Brien D et al. Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. Vaccine 2001; 19(13-14):1732–7.

26. Stegmann T, Morselt HW, Booy FP, van Breemen JF, Scherphof G, Wilschut J. Functional reconstitution of influenza virus envelopes. Embo J 1987;6(9):2651-9.

27. Bron R, Ortiz A, Dijkstra J, Stegmann T, Wilschut J. Preparation, properties, and applications of reconstituted influenza virus envelopes (virosomes). Methods Enzymol 1993;220:313-31.

28. Bungener L, Serre K, Bijl L, Leserman L, Wilschut J, Daemen T et al. Virosome-mediated delivery of protein antigens to dendritic cells. Vaccine 2002;20(17-18):2287-95.

29. Huckriede A, Bungener L, Daemen T, Wilschut J. Influenza virosomes in vaccine development. Methods Enzymol. 2003;373:74-91.

30. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977;83(2):346-56.

31. Miyahira Y, Murata K, Rodriguez D, Rodriguez JR, Esteban M, Rodrigues MM et al. Quantification of antigen specific CD8+ T cells using an ELISPOT assay. J Immunol Methods 1995;181(1):45-54.

32. Verweij WR, de Haan L, Holtrop M, Agsteribbe E, Brands R, van Scharrenburg GJ et al. Mucosal immunoadjuvant activity of recombinant Escherichia coli heat-labile enterotoxin and its B subunit: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen.
Vaccine. 1998;16(20):2069-76.

33. de Haan L, Verweij WR, Holtrop M, Brands R, van Scharrenburg GJ, Palache AM et al. Nasal or intramuscular immunization of mice with influenza subunit antigen and the B subunit of Escherichia coli heat-labile toxin induces IgA- or IgG-mediated protective mucosal immunity. Vaccine. 2001;19(20-22):2898-907.

34. Oxford JS, Manuguerra C, Kistner O, Linde A, Kunze M, Lange W et al. A new European perspective of influenza pandemic planning with a particular focus on the role of mammalian cell culture vaccines. Vaccine. 2005;23(46-47):5440-9.

35. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg (Lond). 1972;70(4):767-77.

36. de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaus AD. Haemagglutination-inhibiting antibody to influenza virus. Dev Biol (Basel). 2003;115:63-73.

37. Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J Clin Microbiol. 1986;24(1):157-60.

38. Geeraedts FCG, Bungener L, Pool J, ter Veer W, Wilschut J, Huckriede A. Whole inactivated influenza vaccine is superior to subunit vaccine in inducing immune responses and secretion of proinflammatory cytokines by DCs. Influenza & Other Respiratory Diseases, in press.

39. Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. Vaccine. 2007;25(18):3554-60.

40. Kistner O, Howard MK, Spruth M, Wodal W, Brühl P, Gerencer M et al. Cell culture (Vero) derived whole virus (H5N1) vaccine based on wild-type virus strain induces cross-protective immune responses. Vaccine 2007;25:6028-36.

41. Hovden AO, Cox RJ, Haaheim LR. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol. 2005;62(1):36-44.

42. Hovden AO, Cox RJ, Madhun A, Haaheim LR. Two doses of parenterally administered split influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral shedding upon challenge in mice. Scand J Immunol. 2005;62(4):342-52

43. Ramisse F, Deramoudt FX, Szatanik M, Bianchi A, Binder P, Hannoun C et al. Effective prophylaxis of influenza A virus pneumonia in mice by topical passive immunotherapy with polyvalent human immunoglobulins or F(ab')2 fragments. Clin Exp Immunol. 1998;111(3):583-7.

44. Belshe RB, Gruber WC, Mendelman PM, Mehta HB, Mahmood K, Reisinger K et al. Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. J Infect Dis. 2000;181(3):1133-7.

45. Moran TM, Park H, Fernandez-Sesma A, Schulman JL. Th2 responses to inactivated influenza virus can be converted to Th1 responses and facilitate recovery from heterosubtypic virus infection.J Infect Dis. 1999;180(3):579-85.

46. Coutelier JP, van der Logt JT, Heessen FW, Warnier G, Van Snick J. IgG2a restriction of murine antibodies elicited by viral infections. J Exp Med. 1987;165(1):64-9.

47. Hocart MJ, Mackenzie JS, Stewart GA. The IgG subclass responses induced by wild-type, cold-adapted and purified haemagglutinin from influenza virus A/Queensland/6/72 in CBA/CaH mice. J Gen Virol. 1988;69 (Pt 8):1873-82.

48. Proietti E, Bracci L, Puzelli S, Di Pucchio T, Sestili P, De Vincenzi E et al. Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. J Immunol. 2002;169(1):375-83.

49. Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA et al. Distinct contribution of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vacc Immunol. 2006;13:981-90.

50. Julkunen I, Hovi T, Seppälä I, Mäkelä O. Immunoglobulin G subclass antibody responses in influenza and parainfluenza type 1 virus infections. Clin Exp Immunol. 1985;60:130-38.

51. Johnson PR, Feldman S, Thompson JM, Mahoney JD, Wright PF. Immunity to influenza A virus infection in young children: a comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. J Infect Dis. 1986;154:121-27.

52. Deng Y, Jing Y, Campbell AE, Gravenstein S. Age-related impaired type 1 T cell responses to influenza: reduced activation ex vivo, decreased expansion in CTL culture in vitro, and blunted response to influenza vaccination in vivo in the elderly. J Immunol. 2004;172(6): 3437-46.

Preservation of the immunogenicity of dry-powder influenza H5N1 whole inactivated virus vaccine at elevated storage temperatures

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Abstract

Stockpiling of pre-pandemic influenza vaccines guarantees immediate vaccine availability to counteract an emerging pandemic. Generally, influenza vaccines need to be stored and handled refrigerated to prevent thermal degradation of the antigenic component. Requirement of a cold-chain, however, complicates stockpiling and the logistics of vaccine distribution. We, therefore, investigated the effect of elevated storage temperatures on the immunogenicity of a pre-pandemic influenza A H5N1 whole inactivated virus vaccine. Either suspended in liquid or kept as a freeze-dried powder, vaccines could be stored for 1 year at ambient temperature (20°C) with minimal loss of immunogenicity in mice. Elevation of the storage temperature to 40°C, however, resulted in a significant loss of immunogenic potency within 3 months if vaccines were stored in liquid suspension. In sharp contrast, freeze-dried powder formulations were stable at 40°C for at least 3 months. The presence of inulin or trehalose sugar excipients during freeze-drying of the vaccine proved to be critical to maintain its immunogenic potency during storage, and to preserve the characteristic Th1-type response to whole inactivated virus vaccine. These results indicate that whole inactivated virus vaccines may be stored and handled at room temperature in moderate climate zones for over a year with minimal decline and, if converted to dry-powder, even in hot climate zones for at least 3 months. The increased stability of dry-powder vaccine at 40°C may also point to an extended shelf-life when stored at 4°C. Use of the more stable dry-powder formulation could simplify stockpiling and thereby facilitating successful pandemic intervention.

Introduction

Animal influenza A viruses that cross the species barrier to humans pose a potential pandemic threat [1]. Generally, these virus subtypes are antigenically different from the circulating human viruses and thus may spread rapidly in the immunologically naïve population, if human-to-human transmission is sustained. Recently, a novel H1N1 influenza virus of combined swine, avian, and human origin became pandemic within two months after its emergence in humans in April 2009 [2, 3]. In terms of virulence this virus is comparable to the H2N2 virus that caused the relatively mild pandemic of 1957 [4-7]. However, a similar pandemic scenario with a more virulent virus subtype like the highly pathogenic avian influenza (HPAI) H5N1 virus would be potentially catastrophic [8, 9]. With a lethality rate of approximately 60% in reported laboratory-confirmed human H5N1 cases [10], maximum preparedness is warranted in case sustained human-to-human transmission were to evolve.

Vaccines and antivirals are critical assets in intervention strategies against pandemic influenza [11, 12]. They represent the only countermeasures that are clearly efficacious in preventing infection and treating illness [13] and their availability plays a central role in pandemic preparedness planning. Prophylactic use of antivirals could provide short-term protection and may be helpful for early containment of an emerging pandemic [14, 15]. However, the control of infection in a pandemic will ultimately depend on protection provided by vaccination.

For the H5N1 virus, effective pre-pandemic vaccines have recently been developed, including whole inactivated virus (WIV) vaccine and split-virus vaccine combined with the oil-in-water emulsion adjuvants AS-03 or MF59 [16-19]. Because the eventual pandemic virus may be an antigenic drift variant of the strain used to produce such pre-pandemic vaccines, a complete match with the pandemic strain cannot be guaranteed. However, the above vaccines have been shown to induce substantial cross-reactive immune responses to viruses of different H5N1 clades [16-18, 20-23]. Moreover, even at suboptimal efficacy pre-pandemic vaccines may reduce attack rates if deployed in an early stage of the pandemic [24, 25] or may serve as priming dose for the true pandemic vaccine, thus saving time and resources [26]. Stockpiling of pre-pandemic H5N1 vaccines is therefore considered an effective tool for containment and mitigation of an emerging pandemic [15], and stockpiles have already been established by multiple countries [27].

Current inactivated influenza vaccine formulations need to be stored refrigerated because the major antigenic component, the viral haemagglutinin (HA), is prone to degradation at higher temperatures [28]. However, cold-chain requirements may seriously complicate the logistics of storage and deployment of pre-pandemic and pandemic vaccines, with a risk of vaccines escaping refrigeration. In general, cold-chain failure and thermal instability is a major cause of vaccine loss in developing countries [29], and has been linked to serious disease outbreaks [30]. Development of temperature-stable vaccines for pandemic use would promote worldwide usability by diminishing the complexity of refrigerated storage and distribution logistics.

A well known strategy to improve stability is conversion of the liquid vaccine into dry powder [31]. Biopharmaceuticals like influenza vaccines are preferably desiccated using freeze-drying techniques [32]. The vaccine is rapidly frozen and water is, subsequently, extracted by sublimation under reduced pressure. Detrimental stresses acting on the HA antigenic components as a result of the freeze-dry process, as well as degradation of the HA during subsequent storage, can be counteracted by the use of cryoprotectants such as specific sugars [33, 34]. Sugar is mixed with the liquid vaccine, and during rapid freezing a glassy sugar matrix is formed in which water and vaccine constituents are captured. The physical barrier separating the vaccine constituents and molecular immobility provided by the matrix prevent the vaccine from degradation. Also, the matrix prevents further crystallization of water molecules upon extended cooling. After drying the vaccine components are incorporated in a dry sugar glass, in which the water has been spatially replaced by sugar and the structural integrity of the vaccine constituents is preserved.

Here, we investigated the effect of ambient and high storage temperatures over time on the immunogenicity of influenza A/Vietnam/1203/2004 (H5N1) whole inactivated virus (WIV) vaccine. Vaccines were either stored conventionally in buffer solution or as sugar-stabilized dry-powder formulations and immunogenicity was assessed in a mouse model. Inulin and trehalose sugars, which have the intrinsic capacity to maintain a glassy state at high temperatures [35, 36], were used as stabilizing agents.

Methods

VACCINE PREPARATION AND STORAGE CONDITIONS

H5N1 virus (NIBRG-14) was provided by the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, UK), and is a 2:6 reassortant between A/Vietnam/1194/2004 (H5N1) and A/PR/8/34 (H1N1) virus produced by reverse genetics technology. WIV was obtained by virus propagation on embryonated chicken eggs, and inactivation of the virus preparation with 0.1% β-propiolactone. The haemagglutinin protein concentration in the vaccines was determined by single radial immunodiffusion (SRID) [37].

Freeze-drying of WIV was performed as described previously [38]. Briefly, glass vials containing a mixture of 200 μl of a 16% w/v sugar (either trehalose or inulin) solution in HBS (2 mM Hepes, 0.15 M NaCl, pH 7.4), or HBS without sugar, and 200 μl WIV (66 μg HA) in HBS (approximated sugar:HA ratio of 500:1 (w/w) were frozen in liquid nitrogen for 10 minutes and subsequently lyophilized in a Christ Alpha 1-4 freeze-dryer (Salm en Kipp, Breukelen, The Netherlands). The setting of the freeze-dryer was -35°C for the shelf temperature, -55°C for the condenser temperature, and 0.220 mbar pressure. After 24 h the pressure was reduced to 0.060 mbar and the shelf temperature was gradually raised to 20°C. This status was maintained for another 24 h. The dried samples were either directly rehydrated for experiments or stored in a silica gel containing desiccator at 20°C or 40°C. The humidity in the dessicator was maintained at the level of 10± 2 % RH. As shown by Hinrichs et al. at 10% RH, trehalose and inulin glasses are equally hygroscopic [39]. After storage vaccines were rehydrated shortly before immunization.

HEMAGGLUTINATION OF ERYTHROCYTES

To determine the particle titer of the vaccines a standard hemagglutination assay was used. Vaccines were serially diluted two fold in PBS in duplicate, starting with a 10 times dilution in the first well of a microtiter plate, so that the end volume per well was 50 μl. An equal volume of a 1% suspension of fresh guinea pig erythrocytes in PBS was added and the plates were incubated at room temperature for 2 h. The titer was determined as the reciprocal of the highest dilution that yielded complete hemagglutination.

HEMOLYSIS OF ERYTHROCYTES

The hemolysis assay was performed as described previously [40]. In short, WIV vaccine (1 μg HA) in 50 μl HNE buffer (5 mM Hepes, 0.15 M NaCl, 0.1 mM EDTA, pH 7.4) was added to $4x10^7$ human erythrocytes in 800 μl HNE. 50 μl fusion buffer (pH 5.5) was added to initiate viral membrane fusion. The total mixture was incubated 0.5 h at 37°C, and subsequently centrifuged at 350 g for 10 minutes. Absorbance of the supernatant was red at 540 nm, representing the haemoglobin liberated from the lysed erythrocytes. The absorbance was corrected for autohemolysis in absence of WIV vaccine. The amount of hemolysis was then given as a percentage of the maximal hemolysis, which was determined by lysing erythrocytes in water.

IMMUNIZATION

For immunization experiments, 8-10-week-old female Balb/c mice were purchased from Harlan Netherlands B.V. (Zeist, The Netherlands). All experiments were conducted with approval of the local Institutional Animal Care and Use Committee. Mice were intramuscularly injected in both their calf muscles with a total of 50 μl WIV vaccine (5 μg HA) in HBS, equally divided over both injection sites. Mouse numbers per immunization group were as follows: liquid WIV; n=5-6, freeze-dried WIV with or without sugar; n=6-8, liquid WIV after 1 year at 20°C; n=3. Twenty eight days after immunization, sera were collected for evaluation.

ELISA

ELISA assays were performed as described previously [41]. Briefly, microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) coated with 0.2 μg influenza H5N1 (NIBRG-14) subunit vaccine per well were blocked with a 2% milk solution. Two-fold serial dilutions of serum samples in 0.05%Tween 20/PBS (PBS/T) were added to the wells in duplicate and incubated for 1.5 h. Bound H5N1 specific IgG, IgG1 or IgG2a antibodies were detected with a horseradish peroxidase-conjugated goat anti-mouse IgG-isotype antibody (Southern Biotech, Birmingham, Alabama). All incubations were performed at 37°C. The staining was performed with o-phenylene-diamine (OPD) (Eastman Kodak Company) and absorbance at 492 nm was measured by an ELISA reader (Bio-tek Instruments, inc.). The antibody titer was calculated by extrapolating the serum dilution corresponding to an OD of 0.2, using linear regression.

Hemagglutination inhibition (HI) assay

The HI assay was performed as described previously [42]. In short, 50 µl kaolin absorbed, heat-inactivated mouse serum in PBS (1:4) was serially diluted two-fold in a microtiter plate in duplicate. Next, 50 µl of 4 hemagglutination units (HAU) of H5N1 (NIBRG-14) virus in PBS was added and incubated for 40 min at RT. Finally, 50 µl of 1% guinea pig erythrocytes (Harlan) in PBS was added to each well and HI titers were determined after 2 h incubation at RT. HI titers are given as the reciprocal of the highest serum dilution producing complete inhibition of hemagglutination.

Statistical analysis

Statistical analysis on antibody titers was performed using the unpaired Student's *t* test. P values of $p<0.05$ and $p<0.01$ were considered statistically significant and highly significant, respectively.

Results

Effect of freeze-drying and reconstitution on vaccine immunogenicity

WIV vaccines were freeze-dried with or without sugar and samples were stored, with WIV vaccine kept in suspension (liquid WIV). Immediately after drying, samples of each vaccine were reconstituted in water and tested alongside liquid WIV, to assess the effect of the actual freeze-drying process on the vaccines, and to obtain reference values for the storage experiments. Evaluation involved assessment of the hemagglutination or hemolytic activities of the vaccines as a measure for the structure and function of the viral particles. Any effect of inulin or trehalose on the hemagglutination and hemolysis assay was excluded, by testing a similar concentration of the freeze-dried sugar without viral particles. The results shown in Table 1 demonstrate that freeze-drying of WIV with or without trehalose or inulin did not have adverse effects on its hemagglutination or hemolytic activities. Preservation of these activities indicates that the sialic-acid-binding capacity and the membrane fusion activity of the HA proteins were retained.

Table I. Effect of High Storage Temperature on Hemagglutination and Hemolysis Activity of the Vaccines

Vaccine	Hemagglutination titer ($10\times^2$log)		Hemolysis (% of max)	
Temperature	–	40°C	–	40°C
Duration	0 Day	3 Months	0 Day	3 Months
Liquid	11	5	67.9	0
FD	≥12	7	90.6	4.3
FDT	11	8	81.5	35.3
FDI	11	8	83.3	15.1

Immunization groups: liquid liquid WIV, FD freeze-dried WIV without sugar, FDT freeze-dried WIV with trehalose, FDI freeze-dried WIV with inulin

To determine whether the preservation of *in vitro* vaccine activity after freeze-drying was accompanied by preservation of *in vivo* vaccine potency, mice were intramuscularly injected with vaccine doses of 5 μg HA. Four weeks after immunization, the antibody responses induced by the freeze-dried vaccines were compared to those induced by liquid WIV. WIV freeze-dried without sugar (FD) induced slightly lower IgG titers (p=0.001) than liquid WIV, while hemagglutination-inhibition (HI) titers were not significantly diminished (p=0.08) (Fig. 1a, b; 0 Day). WIV freeze-dried with trehalose (FDT) was equally immunogenic as liquid WIV, while WIV freeze-dried with inulin (FDI) induced slightly lower IgG titers (p=0.02) but similar HI titers. In conclusion, freeze-drying with or without sugar did not affect the immunogenic potency of H5N1 WIV vaccine substantially.

Effect of storage at ambient temperature on vaccine immunogenicity

The freeze-dried vaccines and liquid WIV were subsequently stored at 20°C. Three months and one year later samples were tested for their capacity to induce antibody responses in mice. After 3 months of storage, no significant differences in serum IgG and HI titers induced by liquid WIV, FDT or FDI vaccines were observed compared to liquid WIV conventionally stored at 4°C (Fig 1a, b; 3 Months). In contrast, FD vaccine induced substantially lower mean IgG and HI titers compared to liquid WIV at 4°C (22.4-fold (p=0.03) and 3.5-fold (p=0.04), respectively (Fig 1a, b; 3 Months). Consequently, FD vaccine was excluded from further evaluation.

After one year of storage at 20°C, liquid WIV and both FDT and FDI still induced considerable H5N1-specific antibody responses. The mean IgG titer induced by liquid WIV stored at 20°C was only slightly, but not significantly (p=0.07), lower than that induced by liquid WIV stored at 4°C (Fig 1a, b; 1 Year), and also the mean HI titer was not significantly altered (Fig 1a, b; 1 Year). IgG titers induced by FDI were moderately lower (2.2 fold, p=0.005) with only a marginal decrease in HI titers. FDT showed a more substantial decrease in both mean IgG and HI titers (p=0.003 and p=0.03, respectively).

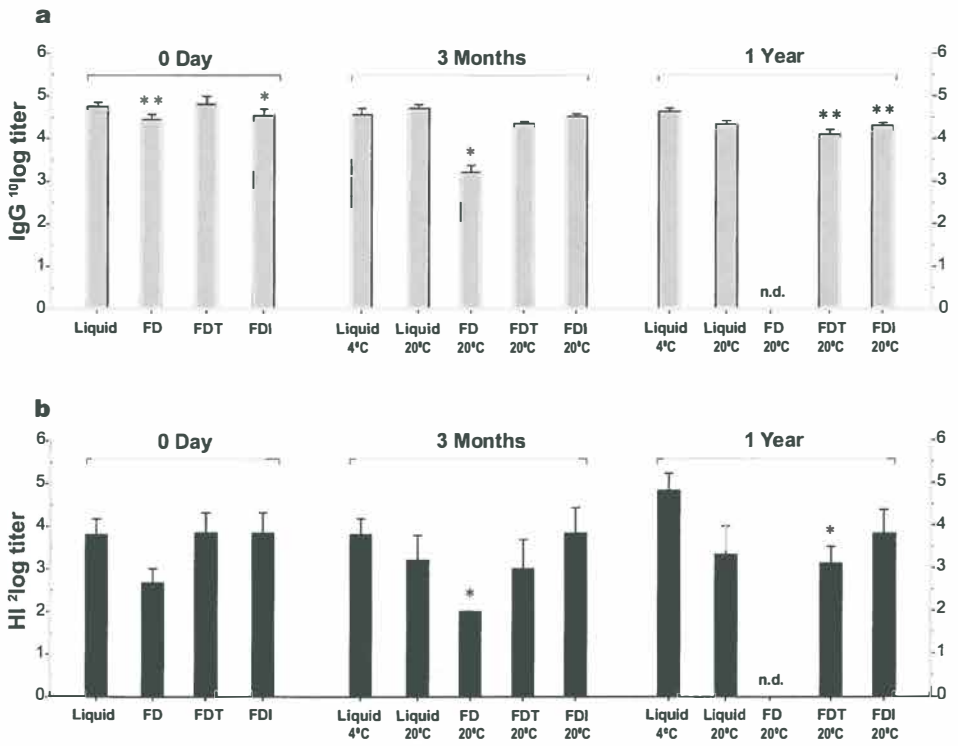

Figure 1. Antibody responses induced by liquid and freeze-dried WIV vaccines before and after storage at ambient temperature. *Serum H5N1-specific IgG titers were determined by ELISA and are given as geometric mean titers + standard error of the means (SEM), before storage, and after 3 months and one year storage (a) . The serum capacity to inhibit agglutination of guinea pig erythrocytes by H5N1 vaccine virus is given in geometric mean HI titers + SEM, before storage, and after 3 months and one year of storage (b). Immunization groups are plotted on the x-axis with the vaccines' actual storage temperature in degrees Celsius: liquid: liquid WIV, FD: freeze-dried WIV without sugar, FDT: freeze-dried WIV with trehalose, FDI: freeze-dried WIV with inulin. HI titers below the detection limit were assigned half the value of the lowest detectable serum dilution, which was 8. * p<0.05, ** p<0.01. Nd; not determined.*

Because ELISA and HI results obtained at different time points may be subject to inter-assay variation, (pooled) sera from all immunization experiments were tested in a single assay, in order to compare the immunogenicity of the vaccines over time (Fig 2). Clearly, at 20°C the effect of storage duration on the immunogenic potency of the vaccine was minimal for liquid WIV and FDT and FDI vaccine. IgG titers dropped maximally by a factor of 1.9 (liquid WIV), and the maximum effect on HI titers was a 2-fold reduction (FDT and FDI), after 1 year of storage (Fig 2a/b). More than 2-fold reductions in IgG or HI titers were only seen for FD vaccine after 3 months storage. In conclusion, liquid WIV and sugar stabilized freeze-dried WIV revealed only minor reductions in the immunogenicity after one year of storage at 20°C. At this condition substantial deterioration of immunogenicity of WIV vaccine was seen only for WIV freeze-dried in absence of sugar excipient.

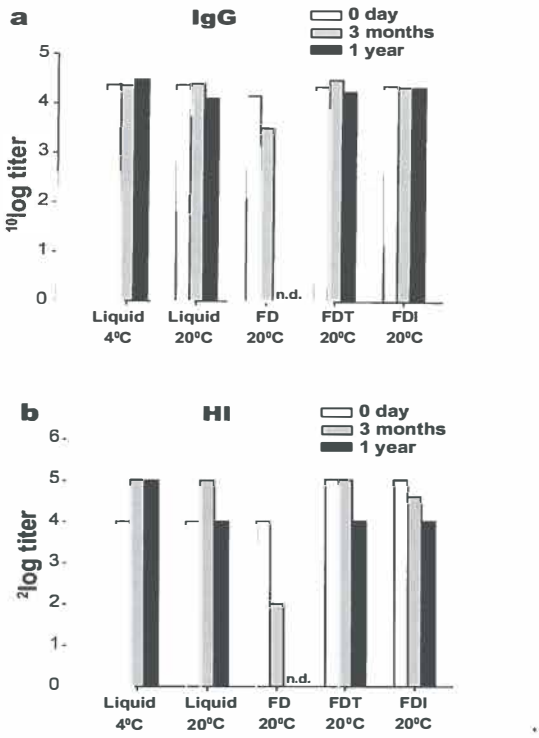

Figure 2. Effect of storage duration on the vaccines capacity to induce antibody responses. *Pooled sera of each group of mice immunized at different time points with liquid or freeze-dried WIV vaccine were tested simultaneously on H5N1-specific IgG or hemagglutination inhibiting capacity. Bars represent the average values of duplicate determinations (deviations were less than 0.2). Immunization groups are plotted and labelled as in Fig. 1. Storage duration: 0 day (white bars), 3 months (grey bars), 1 year (black bars). Nd; not determined.*

EFFECT OF HIGH STORAGE TEMPERATURE ON VACCINE IMMUNOGENICITY

To simulate more challenging conditions vaccines may encounter during storage and transportation, the vaccines were put on shelf at 40°C for 3 months. These conditions negatively affected the hemagglutination and hemolysis activity of all vaccines, but strongest for liquid WIV and FD vaccine (Table 1). Subsequent immunization of mice revealed a strong decrease in IgG and HI titers induced by liquid WIV stored at 40°C, compared to WIV freshly prepared from a frozen virus stock (Fig 3a, b). The immunogenic potency of vaccines freeze-dried with sugars was much better preserved under these challenging conditions. For FDT vaccine a slight decrease in mean IgG titer (1.4 fold, p=0.08) together with a more substantial decrease in HI titer (2.3 fold, p=0.01) was found. Responses to FDI vaccine were not significantly different from those to freshly prepared vaccine (Fig 3a, b). In contrast, freeze-drying without additional measures to stabilize the vaccine resulted in strongly reduced IgG and HI titers after storage at 40°C. Compared to liquid WIV stored at 40°C , both FDT and FDI stored at 40°C induced significantly higher IgG titres (p=0.002 and p=0.00001, respectively), and HI titres (p=0.002 and p= 0.02, respectively), which was not the case for FD vaccine (Fig 3a, b).

The effect of high storage temperature relative to ambient storage temperature on vaccine immunogenicity is summarized in Figure 4. Serum pools of the different immunization groups were run in a single assay, and the IgG and HI titers were plotted including those induced by liquid WIV stored at 4°C as a reference. The deteriorating effect of increasing temperature was most explicit for liquid WIV and FD vaccine. In case of FD formulation, maximum deterioration was already observed at moderate temperatures. If stored as FDT or FDI formulation, however, vaccines remained stable independent of the storage temperature.

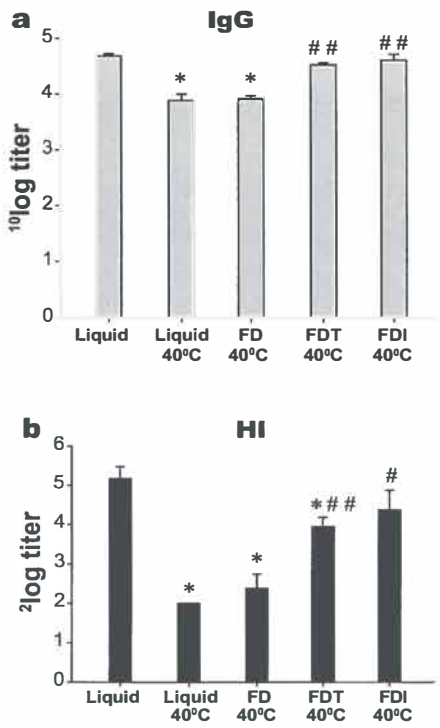

Figure 3. Effect of high storage temperature on the immunogenic potency of the vaccines. *Vaccines were stored for 3 months at 40°C and subsequently injected in mice. Serum H5N1-specific IgG titers (a) and HI titers (b) were determined, and are shown as geometric mean titers + SEM. Immunization groups are labelled as in Fig. 1. The group immunized with liquid WIV, which had not been subjected to the storage experiment, serves as a standard. * $p<0.05$, when compared with the standard. # $p<0.05$, ## $p<0.01$, when compared with liquid WIV stored at 40°C.*

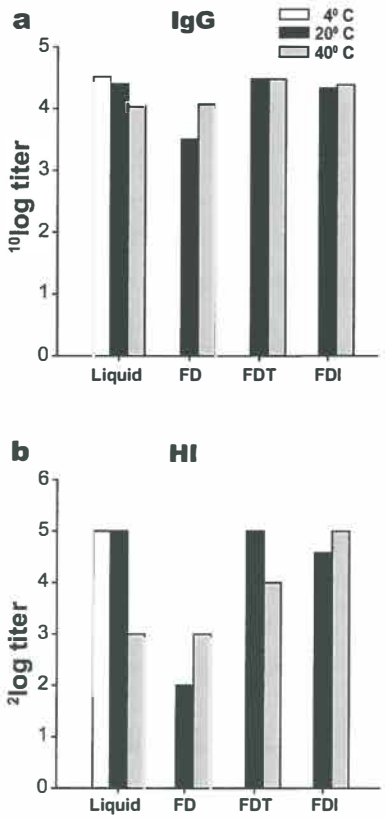

Figure 4. Effect of storage temperature on the vaccines' capacity to induce antibody responses. *Pooled sera of each group of mice immunized with liquid or freeze-dried WIV vaccine, which had been stored for 3 months at different temperatures, were tested simultaneously on H5N1-specific IgG or HI capacity. Bars represent the average values of duplicate determinations (deviations were less than 0.2). Immunization groups as in Fig 1. Storage temperatures: 4°C (white bars), 20°C (grey bars), 40°C (black bars).*

PRESERVATION OF THE QUALITY OF THE IMMUNE RESPONSE TO WIV

The ratio of IgG2a and IgG1 subtype antibodies induced by the vaccine is important for protective efficacy. Relatively high IgG2a titers are representative of the Th1 type response characteristically induced by whole virus particles [42, 43] and are associated with better protection against a challenge with homologous virus in mice [44]. We, therefore, evaluated the effect of freeze-drying and storage of the vaccine on the phenotype of the induced antibody response. When WIV was stored in buffer solution, the IgG2a-dominated antibody response was preserved at all storage temperatures and storage durations tested (Table 2.). Freeze-drying in absence of a stabilizing sugar excipient reduced the IgG2a/IgG1 ratio to the

level of a mixed IgG2a/IgG1 phenotype. Subsequent storage of the FD vaccine further lowered the IgG2a/IgG1 ratio resulting in a shift to a IgG1-dominated antibody response within 3 months, independent of the storage temperature. In contrast, FDT and FDI vaccine induced an IgG2a-dominated antibody response after storage independent of storage duration and temperature.

Table II. Dominance of IgG Subtypes in the Antibody Response to Vaccination. Immunization Groups are Labeled as in Table I

Vaccine		0 months	3 months	12 months
Liquid	4°C	lgG2a[a]	lgG2a	lgG2a
	20°C	nd	lgG2a	lgG2a
	40°C	nd	lgG2a	nd
FD	20°C	lgG1/lgG2a[b]	lgG1	nd
	40°C	nd	lgG1	nd
FDT	20°C	lgG2a	lgG2a	lgG2a
	40°C	nd	lgG2a	nd
FDI	20°C	lgG2a	lgG2a	lgG2a
	40°C	nd	lgG2a	nd

nd not done

a IgG2a or IgG1 were defined dominant if the IgG2a/IgG1 ratio was ≥1.5 or ≤0.5, respectively

b For ratios between 0.5 and 1.5, the response was defined as mixed

Discussion

The immunogenicity of WIV vaccine depends on the presence and integrity of the HA proteins and an intact viral particle structure [42, 45]. The HA antigens provide the B-cell epitopes and define the specificity of the antibody response, while the viral particle acts as a vehicle for the vaccines primary immunopotentiating agent, the viral single-stranded RNA (ssRNA). Its immunopotentiating activity is exerted through binding to Toll-like receptor 7 (TLR7) in the endosomes of recipient host cells, and triggering of the innate immune system [41, 46, 47]. To a large extent, this mechanism accounts for the superior immunogenicity of WIV compared to split-virus formulations or subunit vaccines, which do not contain intact viral RNA [41]. TLR7 signaling also determines the response type, being characteristically of a Th1 type for WIV, with a high amount of IgG2a/c antibodies in mice.

In liquid WIV vaccine, the antigenic properties of HA and the immunopotentiating activity provided by the ssRNA appear to be remarkably stable. Our results show that the immunogenicity of WIV vaccine stored in buffer solution at 20°C remained well preserved with minimal loss of activity over a period of at least 12 months. In contrast, seasonal subunit vaccine (H3N2) stored in buffer solution at ambient (25°C) temperature was reported to lose potency already over a period of 12 to 20 weeks [28]. Compared to subunit vaccine, WIV, therefore, appears to be more stable. Yet, it should be noted that the comparability of the two studies is limited since they differ in the virus strains used for vaccine production and the method by which vaccine stability was assessed (in vivo immunogenicity versus single radial immunodiffusion assay (SRID) [31]). At high storage temperature (40°C), the immunogenic potency of liquid WIV rapidly deteriorated. Progressive degradation of HA antigens and/or loss of intact viral particles, as reflected in the strongly reduced hemagglutination and hemolytic activity of the vaccine, may likely be the cause. Yet, the antibody response remained Th1 skewed, which may indicate that a small amount of viral particles escaped degradation, as it was shown previously that even a very low dose of viral particles is sufficient for Th1 skewing of the response to WIV vaccine [44].

At high storage temperature dry-powder formulations were superior to liquid WIV, as reported by others [29, 48]. With the use of sugar stabilization no substantial loss of immunogenicity was observed after storage of freeze-dried WIV for 3 months at 40°C. Furthermore, sugar stabilization played a critical role in preserving the Th1-skewing capacity of the vaccine during freeze-drying and subsequent storage. Freeze-drying in absence of sugar led to a mixed Th1/Th2 antibody response, which further shifted during storage at elevated temperature to an overt Th2 type response, while the characteristic Th1 phenotype of the WIV response was retained when sugars were used during freeze-drying. Sugar molecules obviously play a role in preserving effective TLR7 signaling by the ssRNA, probably by stabilizing the viral particle structure and protecting the viral ssRNA from degradation. Sugars are known to stabilize enveloped viruses during freeze-drying [49, 50] and trehalose and inulin have the capacity to stabilize lipid bilayers [35, 36, 51]. In freeze-drying experiments with 'empty' viral envelopes (virosomes), use of inulin was found to preserve the vesicular structure, while absence of sugar stabilization resulted in complete disintegration after rehydration [38]. The mode of action of trehalose and inulin is presumably by replacement of the water molecules situated in between the hydrophilic heads of the lipids.

Hereby, a detrimental phase transition of the viral membrane upon rehydration is prevented and the vaccine particles are preserved [35].

By assessing immunogenicity *in vivo* we discovered an important role for sugar compounds in preserving not only the quantity but also the quality of the immune response to WIV after freeze-drying and storage. Previous storage studies that showed improved stability of freeze-dried WIV used the hemagglutination assay to determine vaccine stability, which provides a quantitative measure only [29, 33]. The shift from a Th1 response to WIV before freeze-drying to a Th2 response to WIV after freeze-drying in absence of sugar-stabilization could not have been predicted from hemagglutination results, nor by other in vitro tests that assess the integrity of the HA component, like the SRID. *In vitro* HA stability tests alone are therefore insufficient to obtain a complete representation of WIV vaccine stability.

The efficacy of influenza vaccines is determined by the induction of an effective antibody response. The serum HI titre, which is a measure for the magnitude of the antibody response, is the principle correlate of protection used to evaluate the efficacy of current human seasonal and pre-pandemic influenza vaccines. For seasonal vaccines an HI titre of >40 is considered to be protective [52-55] . For the mouse model no protective titre has been defined. Due to the intrinsic low immunogenicity of H5N1 virus and the single-dose immunization scheme the maximal titre reached in our experiments was 32. It has to be noted that immunogenicity in terms of HI and IgG titers does not necessarily correlate with protective efficacy. In the mouse model, the relative amounts of IgG1 and IgG2a (or IgG2c), expressed in the IgG2a/IgG1 ratio, appear to be decisive for protection [40, 56, 57] IgG2a-dominated responses are clearly more effective than IgG1-dominated responses. Yet, in the absence of significant differences in HI and IgG titres and similar IgG subtype dominance, as observed for example for liquid WIV, FDI and FDT during storage at ambient temperatures, protective efficacy is likely to be comparable.

The preservability of WIV vaccine is strongly increased by sugar stabilization and freeze-drying. Similarly, other formulations like subunit, split-virus and virosome [31] vaccines have been successfully freeze-dried and stabilized with sugars (summarized in [31]). Yet, a combination of increased stability together with specific features of WIV which promote vaccine availability, like strong immunogenicity, dose sparing quality and manufacturing simplicity [46], make dry-powder WIV an apt candidate formulation for pre-pandemic

stockpiling. Based on the results of storage at 40°C it may also be predicted that dry-powder WIV will have a shelf-life exceeding that of liquid WIV, when kept under optimal refrigerated conditions [52, 58]. An extended vaccine shelf-life could delay costly replacement of stocks when vaccines reach their expiry date. In case of a pandemic emergency, deployment of dry-powder vaccine stocks may be exerted without refrigeration unless the temperatures are extremely high. This could speed up pandemic intervention and reduce losses due to cold-chain failures.

Conclusion

The lack of suitable vaccines during the initial emergence of the New Influenza A (H1N1)swl pandemic virus in 2009, and its rapidly global spread underscores the importance of effective pre-pandemic vaccine stockpiles. Although this virus was of an unforeseen subtype, it remains important that stockpiles of vaccines against identified potentially dangerous virus strains like the H5N1 virus are prepared and kept in place. Our results suggest that for stockpiling liquid H5N1 WIV vaccine refrigeration may not be an absolute requirement to preserve the vaccines immunogenicity, as long as the temperature remains below 20°C. Furthermore, conversion from liquid to dry-powder formulation increases the versatility of WIV vaccine by allowing storage outside the fridge at higher temperatures, up to 40°C for at least 3 months. Because of its improved stability, use of sugar-stabilized freeze-dried H5N1 WIV vaccine could increase the efficacy of pre-pandemic stockpiling and subsequent vaccine deployment.

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References

1. N.P. Johnson, and J. Mueller. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med 76, 105-15 (2002).
2. http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html.
3. F.S. Dawood, S. Jain, L. Finelli, M.W. Shaw, S. Lindstrom, R.J. Garten, L.V. Gubareva, X. Xu, C.B. Bridges, and T.M. Uyeki. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 360, 2605-15 (2009).
4. Update: novel influenza A (H1N1) virus infections - worldwide, May 6, 2009. MMWR Morb Mortal Wkly Rep 58, 453-8 (2009).
5. C. Fraser, C.A. Donnelly, S. Cauchemez, W.P. Hanage, M.D. Van Kerkhove, T.D. Hollingsworth, J. Griffin, R.F. Baggaley, H.E. Jenkins, E.J. Lyons, T. Jombart, W.R. Hinsley, N.C. Grassly, F. Balloux, A.C. Ghani, N.M. Ferguson, A. Rambaut, O.G. Pybus, H. Lopez-Gatell, C.M. Alpuche-Aranda, I.B. Chapela, E.P. Zavala, D.M. Guevara, F. Checchi, E. Garcia, S. Hugonnet, and C. Roth. Pandemic potential of a strain of influenza A (H1N1): early findings. Science 324, 1557-61 (2009).
6. http://www.who.int/csr/don/2009_06_12/en/index.html.
7. J.S. Nguyen-Van-Tam, and A.W. Hampson. The epidemiology and clinical impact of pandemic influenza. Vaccine 21, 1762-8 (2003).
8. D.S. Fedson. Vaccine development for an imminent pandemic: why we should worry, what we must do. Hum Vaccin 2, 38-42 (2006).
9. J.K. Taubenberger, A.H. Reid, R.M. Lourens, R. Wang, G. Jin, and T.G. Fanning. Characterization of the 1918 influenza virus polymerase genes. Nature 437, 889-93 (2005).
10. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_06_02/en/index.html.
11. http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_GIP_2005_5/en/.
12. L. Uscher-Pines, S.B. Omer, D.J. Barnett, T.A. Burke, and R.D. Balicer. Priority setting for pandemic influenza: an analysis of national preparedness plans. PLoS Med 3, e436 (2006).
13. A.S. Monto. Vaccines and antiviral drugs in pandemic preparedness. Emerg Infect Dis 12, 55-60 (2006).
14. F.G. Hayden, and A.T. Pavia. Antiviral management of seasonal and pandemic influenza. J Infect Dis 194 Suppl 2, S119-26 (2006).
15. I.M. Longini, Jr., A. Nizam, S. Xu, K. Ungchusak, W. Hanshaoworakul, D.A. Cummings, and M.E. Halloran. Containing pandemic influenza at the source. Science 309, 1083-7 (2005).

16. H.J. Ehrlich, M. Muller, H.M. Oh, P.A. Tambyah, C. Joukhadar, E. Montomoli, D. Fisher, G. Berezuk, S. Fritsch, A. Low-Baselli, N. Vartian, R. Bobrovsky, B.G. Pavlova, E.M. Pollabauer, O. Kistner, and P.N. Barrett. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N Engl J Med 358, 2573-84 (2008).

17. I. Leroux-Roels, A. Borkowski, T. Vanwolleghem, M. Drame, F. Clement, E. Hons, J.M. Devaster, and G. Leroux-Roels. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet 370, 580-9 (2007).

18. K. Levie, I. Leroux-Roels, K. Hoppenbrouwers, A.D. Kervyn, C. Vandermeulen, S. Forgus, G. Leroux-Roels, S. Pichon, and I. Kusters. An adjuvanted, low-dose, pandemic influenza A (H5N1) vaccine candidate is safe, immunogenic, and induces cross-reactive immune responses in healthy adults. J Infect Dis 198, 642-9 (2008).

19. J. Lin, J. Zhang, X. Dong, H. Fang, J. Chen, N. Su, Q. Gao, Z. Zhang, Y. Liu, Z. Wang, M. Yang, R. Sun, C. Li, S. Lin, M. Ji, X. Wang, J. Wood, Z. Feng, Y. Wang, and W. Yin. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet 368, 991-7 (2006).

20. B. Baras, K.J. Stittelaar, J.H. Simon, R.J. Thoolen, S.P. Mossman, F.H. Pistoor, G. van Amerongen, M.A. Wettendorff, E. Hanon, and A.D. Osterhaus. Cross-protection against lethal H5N1 challenge in ferrets with an adjuvanted pandemic influenza vaccine. PLoS One 3, e1401 (2008).

21. G. Fazekas, R. Martosne-Mendi, I. Jankovics, I. Szilvasy, and Z. Vajo. Cross-reactive immunity to clade 2 strains of influenza virus A subtype H5N1 induced in adults and elderly patients by Fluval, a prototype pandemic influenza virus vaccine derived by reverse genetics, formulated with a phosphate adjuvant, and directed to clade 1 strains. Clin Vaccine Immunol 16, 437-43 (2009).

22. E.A. Govorkova, R.J. Webby, J. Humberd, J.P. Seiler, and R.G. Webster. Immunization with reverse-genetics-produced H5N1 influenza vaccine protects ferrets against homologous and heterologous challenge. J Infect Dis 194, 159-67 (2006).

23. S. Murakami, A. Iwasa, K. Iwatsuki-Horimoto, M. Ito, M. Kiso, H. Kida, A. Takada, C.A. Nidom, Q. Mai le, S. Yamada, H. Imai, Y. Sakai-Tagawa, Y. Kawaoka, and T. Horimoto. Cross-clade protective immunity of H5N1 influenza vaccines in a mouse model. Vaccine 26, 6398-404 (2008).

24. N.M. Ferguson, D.A. Cummings, S. Cauchemez, C. Fraser, S. Riley, A. Meeyai, S. Iamsirithaworn, and D.S. Burke. Strategies for containing an emerging influenza pandemic in Southeast Asia. Nature 437, 209-14 (2005).

25. S. Riley, J.T. Wu, and G.M. Leung. Optimizing the dose of pre-pandemic influenza vaccines to reduce the infection attack rate. PLoS Med 4, e218 (2007).

26. N.A. Goji, C. Nolan, H. Hill, M. Wolff, D.L. Noah, T.B. Williams, T. Rowe, and J.J. Treanor. Immune responses of healthy subjects to a single dose of intramuscular

inactivated influenza A/Vietnam/1203/2004 (H5N1) vaccine after priming with an antigenic variant. J Infect Dis 198, 635-41 (2008).

27. L.C. Jennings, A.S. Monto, P.K. Chan, T.D. Szucs, and K.G. Nicholson. Stockpiling prepandemic influenza vaccines: a new cornerstone of pandemic preparedness plans. Lancet Infect Dis 8, 650-8 (2008).
28. F. Coenen, J.T. Tolboom, and H.W. Frijlink. Stability of influenza sub-unit vaccine. Does a couple of days outside the refrigerator matter? Vaccine 24, 525-31 (2006).
29. J. Huang, R.J. Garmise, T.M. Crowder, K. Mar, C.R. Hwang, A.J. Hickey, J.A. Mikszta, and V.J. Sullivan. A novel dry powder influenza vaccine and intranasal delivery technology: induction of systemic and mucosal immune responses in rats. Vaccine 23, 794-801 (2004).
30. B.D. Schoub, and N.A. Cameron. Problems encountered in the delivery and storage of OPV in an African country. Dev Biol Stand 87, 27-32 (1996).
31. J.P. Amorij, A. Huckriede, J. Wilschut, H.W. Frijlink, and W.L. Hinrichs. Development of stable influenza vaccine powder formulations: challenges and possibilities. Pharm Res 25, 1256-73 (2008).
32. W. Wang. Lyophilization and development of solid protein pharmaceuticals. Int J Pharm 203, 1-60 (2000).
33. J.P. Amorij, J. Meulenaar, W.L. Hinrichs, T. Stegmann, A. Huckriede, F. Coenen, and H.W. Frijlink. Rational design of an influenza subunit vaccine powder with sugar glass technology: preventing conformational changes of haemagglutinin during freezing and freeze-drying. Vaccine 25, 6447-57 (2007).
34. Y.F. Maa, M. Ameri, C. Shu, L.G. Payne, and D. Chen. Influenza vaccine powder formulation development: spray-freeze-drying and stability evaluation. J Pharm Sci 93, 1912-23 (2004).
35. L.M. Crowe, J.H. Crowe, A. Rudolph, C. Womersley, and L. Appel. Preservation of freeze-dried liposomes by trehalose. Arch Biochem Biophys 242, 240-7 (1985).
36. I.J. Vereyken, V. Chupin, F.A. Hoekstra, S.C. Smeekens, and B. de Kruijff. The effect of fructan on membrane lipid organization and dynamics in the dry state. Biophys J 84, 3759-66 (2003).
37. M.S. Williams, R.E. Mayner, N.J. Daniel, M.A. Phelan, S.C. Rastogi, F.M. Bozeman, and F.A. Ennis. New developments in the measurement of the hemagglutinin content of influenza virus vaccines by single-radial-immunodiffusion. J Biol Stand 8, 289-96 (1980).
38. J. de Jonge, J.P. Amorij, W.L. Hinrichs, J. Wilschut, A. Huckriede, and H.W. Frijlink. Inulin sugar glasses preserve the structural integrity and biological activity of influenza virosomes during freeze-drying and storage. Eur J Pharm Sci 32, 33-44 (2007).
39. W.L. Hinrichs, M.G. Prinsen, and H.W. Frijlink. Inulin glasses for the stabilization of therapeutic proteins. Int J Pharm 215, 163-74 (2001).

40. T. Stegmann, H.W. Morselt, F.P. Booy, J.F. van Breemen, G. Scherphof, and J. Wilschut. Functional reconstitution of influenza virus envelopes. EMBO J 6, 2651-9 (1987).

41. F. Geeraedts, N. Goutagny, V. Hornung, M. Severa, A. de Haan, J. Pool, J. Wilschut, K.A. Fitzgerald, and A. Huckriede. Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling. PLoS Pathog 4, e1000138 (2008).

42. F. Geeraedts, L. Bungener, J. Pool, W. ter Veer, J. Wilschut, and A. Huckriede. Whole inactivated virus influenza vaccine is superior to subunit vaccine in inducing immune responses and secretion of proinflammatory cytokines by DCs. Influenza Other Respi Viruses 2, 41-51 (2008).

43. A.O. Hovden, R.J. Cox, and L.R. Haaheim. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol 62, 36-44 (2005).

44. L. Bungener, F. Geeraedts, W. Ter Veer, J. Medema, J. Wilschut, and A. Huckriede. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine 26, 2350-9 (2008).

45. N. Hagenaars, E. Mastrobattista, H. Glansbeek, J. Heldens, H. van den Bosch, V. Schijns, D. Betbeder, H. Vromans, and W. Jiskoot. Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model. Vaccine 26, 6555-63 (2008).

46. http://www.who.int/vaccines-documents/DocsPDF06/863.pdf.

47. S.S. Diebold, T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529-31 (2004).

48. R.J. Garmise, H.F. Staats, and A.J. Hickey. Novel dry powder preparations of whole inactivated influenza virus for nasal vaccination. AAPS PharmSciTech 8, E81 (2007).

49. J.A. Levy, and A.H. Fieldsteel. Freeze-drying is an effective method for preserving infectious type C retroviruses. J Virol Methods 5, 165-71 (1982).

50. D.A. Yannarell, K.M. Goldberg, and R.N. Hjorth. Stabilizing cold-adapted influenza virus vaccine under various storage conditions. J Virol Methods 102, 15-25 (2002).

51. D.K. Hincha, E. Zuther, E.M. Hellwege, and A.G. Heyer. Specific effects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying. Glycobiology 12, 103-10 (2002).

52. http://www.ich.org/LOB/media/MEDIA419.pdf

53. S. Koyama, K.J. Ishii, H. Kumar, T. Tanimoto, C. Coban, S. Uematsu, T. Kawai, and S. Akira. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol 179, 4711-20 (2007).

54. D. Hobson, R.L. Curry, A.S. Beare, and A. Ward-Gardner. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg (Lond) 70, 767-77 (1972).

55. P.M. Kendal AP, Skehel J. Concepts and procedures for laboratory-based influenza surveillance. . Centers for Disease Control, Atlanta, GA. Publication No. B17-35. , (1982).

56. A.O. Hovden, R.J. Cox, A. Madhun, and L.R. Haaheim. Two doses of parenterally administered split influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral shedding upon challenge in mice. Scand J Immunol 62, 342-52 (2005).

57. V.C. Huber, R.M. McKeon, M.N. Brackin, L.A. Miller, R. Keating, S.A. Brown, N. Makarova, D.R. Perez, G.H. Macdonald, and J.A. McCullers. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol 13, 981-90 (2006).

58. P.R. Krause. Goals of stability evaluation throughout the vaccine life cycle. Biologicals 37, 369-78; discussion 421-3 (2009).

General Discussion Part I

Influenza vaccines – what do we want and how can we get it?

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Abstract

Influenza vaccines have been in use since more than 60 years and have proven to be efficacious in protecting from influenza infections during epidemics and the recent H1N1 pandemic. Yet, the development of influenza vaccines has so far been largely based on empirical grounds, which leaves room for vaccine improvement by implementation of recent insights in innate and adaptive immunity. Also, evaluation and approval of new vaccines relies on rather broad correlates of protection like the hemagglutination inhibition titer thereby neglecting qualitative aspects of the immune response. Here we discuss how current inactivated influenza vaccine formulations differ in the type of immune response they elicit and in their protective capacity and what causes these differences. Finally, we will discuss how this knowledge can guide the development of new adjuvants which optimize the protective efficacy of influenza vaccines.

Introduction

Influenza A virus is a highly transmissible infectious agent responsible for 500.000 deaths in annual epidemics and excess deaths during sporadically occurring pandemics [1]. The most dramatic example is the Spanish flu in 1918, with an estimated death toll of over 50 million worldwide [2]. Infection with influenza virus can be asymptomatic but usually produces symptoms, classically fever, cough and other respiratory symptoms. Especially during pandemics more atypical and severe symptoms may be seen, like diarrhea and life threatening viral pneumonia [3,4].

Influenza infections induce vigorous immune responses comprising antibodies of the IgA and IgG subclass, T helper 1 (Th1) cells and cytotoxic T lymphocytes [5-7]. Nevertheless, re-infections are common due to rapid mutational changes of antigenic epitopes on the major influenza virus surface antigen, hemagglutinin (HA). Such mutations, caused by the poor fidelity of the viral RNA polymerase, lead to 'antigenic drift' and are considered the reason for the occurrence of yearly influenza epidemics. The genome of influenza A virus is built up of 8 segments and antigenic variation may take a leap when whole segments of the viral genome change. Such a change of the HA-encoding segment is associated with 'antigenic shift' and may lead to new virus strains which can spread easily in an immunologically naïve human population thus causing a pandemic. Segment changes may be the result of reassortment of genomic segments between circulating viruses and other subtype viruses from different species, like avian influenza. Such reassorted viruses caused the 1957 Asian Flu and the 1968 Hong Kong Flu [8]. Alternatively, a virus subtype circulating in animal species and carrying an HA subtype that differs strongly from those of human virus strains may cross the species barrier and start a pandemic if sustained human-to-human transmission evolves. Such zoönoses were the causes of the Spanish Flu and the 2009 H1N1 pandemic [9,10]. Also the avian H5N1 virus is a zoönotic virus which regularly crosses the species barrier. Due to its very high mortality (about 60% of laboratory-confirmed cases) H5N1 poses a serious threat [11], yet human-to-human transmission so far occurs only very sporadically if at all [12].

Vaccination has proven to be highly efficacious in preventing influenza during epidemic periods and is considered as the most promising mitigation strategy in case of a pandemic [13]. Yet, current epidemic vaccines still suffer from

limited immunogenicity in important risk populations like the elderly and immuno-compromised individuals. Pandemic vaccines, on the other hand, need to be highly immunogenic at low antigen dose in order to protect a very large population against a new virus, with limited vaccine production capacities at hand. So far, vaccine design has been largely empirical. A more rational approach to influenza vaccine development starts with a good understanding of immune responses evoked by current vaccines and the immune mechanisms involved in raising these responses. These issues and their impact on the development of new vaccines will be discussed below.

Current influenza vaccines

Current inactivated influenza vaccines consist of either preparations of whole virus (WIV), detergent-treated virus (split-virus), isolated viral surface proteins (subunit) or reconstituted viral membranes (virosomes) (Fig. 1). With few exceptions (see below) these vaccines are used without adjuvant. Split-virus (SV) and subunit (SU) vaccines have largely replaced the archetype WIV vaccines in the 1960s and 1970s, because of their lower reactogenicity [14]. Yet, modern production technology might alleviate the reactogenicity problem of WIV vaccines and therefore these vaccines have received renewed attention as (candidate) formulations in the context of a putative H5N1 pandemic and the current H1N1 pandemic [15-18].

Vaccine efficacy is primarily defined by the levels of antibodies induced against HA, which is considered the minimally required constituent of an influenza vaccine. Antibody levels are typically measured by determination of the hemagglutination inhibition (HI) titer, the dilution at which serum of a vaccinee is still capable of inhibiting hemagglutination of erythrocytes by the virus. An HI titer of 40 is estimated to be associated with a 50% reduction of the risk of contracting influenza. This titer is used as the basis for approval of influenza vaccines by the European Medicines Agency (EMEA) and the Food and Drug Administration (FDA) [19]. The 50% protective titer was calculated from a number of clinical studies in which immunity was achieved by either natural infection or by vaccination with inactivated or live-attenuated influenza vaccines. Since infection as well as vaccination will induce a plethora of immune reactions, it is unclear whether HI antibodies themselves provide protection or whether their presence is simply an indication for the immune status to influenza virus [20].

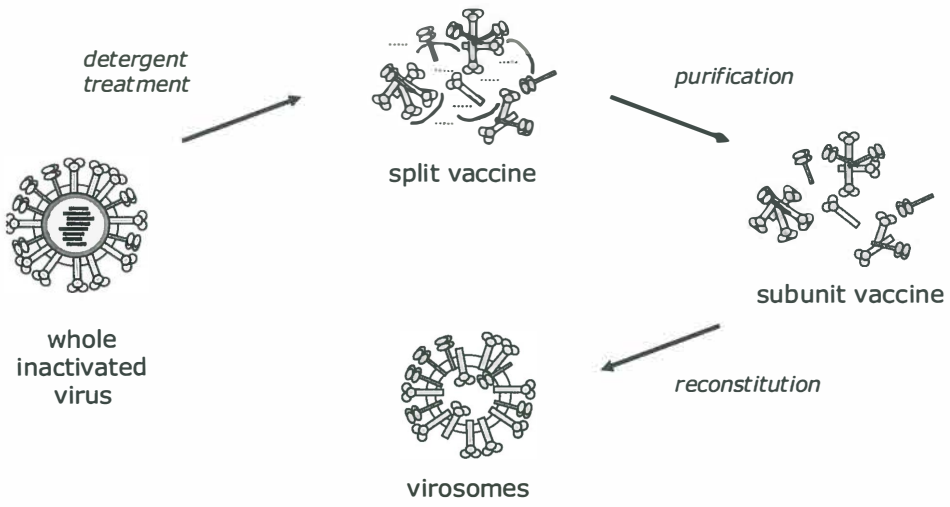

Figure 1. Current inactivated influenza vaccines. *Egg- or cell-grown virus is chemically inactivated to obtain WIV vaccine. Solubilization of the viral membrane by detergent treatment results in SV vaccine. By further purification steps SU vaccine is obtained which contains only the viral membrane proteins. These can be reconstituted with natural or synthetic lipids to empty virus envelopes, so-called virosomes.*

In primed individuals, unadjuvanted WIV, SV, and SU vaccines in general induce similar immune responses in terms of hemagglutination inhibition (HI) titers (for a meta-analysis over 24 studies see [21]). However, in individuals that have not been exposed to the vaccine antigens before, WIV vaccines are more immunogenic than SV and SU vaccines [21-23]. Among inactivated, unadjuvanted H5N1 candidate vaccines only WIV (derived from wild-type virus) evoked antibody titers which met the EMEA and FDA criteria [24]. A cell culture-derived WIV mock-up vaccine, Celvapan®, was licensed in Europe in 2009 and an H1N1 Celvapan® vaccine has been used during the 2009 H1N1 pandemic.

No data are available on the relative protective efficacy of WIV, SV and SU influenza vaccines in humans. Yet, in animal models a head-to-head comparison of different vaccines in terms of their protective capacity can be performed. Challenge studies in mice show that a single immunization with WIV protects more efficiently from weight loss (Fig. 2) and leads to a stronger decrease in nose or lung virus titer than immunization with other vaccines (ref. [25], and Chapter 5). Moreover, WIV prevents weight loss and reduces viral loads in the lung

already at low antigen doses, whereas substantially higher doses of SV and SU vaccine are needed to achieve the same effect [26].

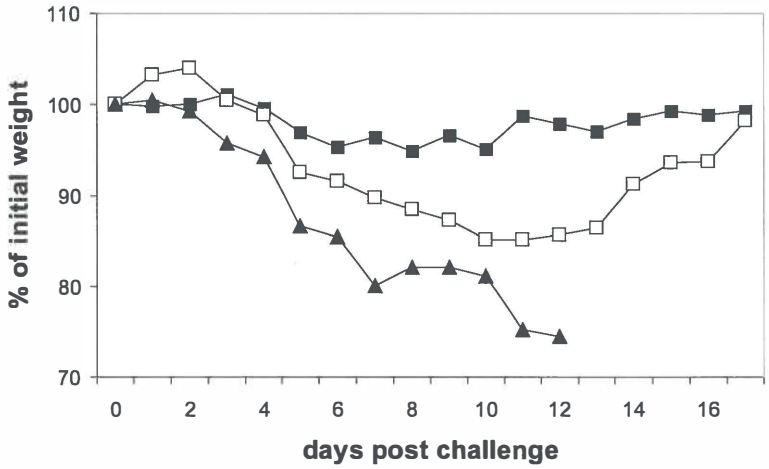

Figure 2. Protection from influenza challenge by different vaccines. *Mice were immunized intramuscularly on day 0 with 5 μg HA formulated as WIV vaccine (filled squares) or SU vaccine (open squares), or were injected with buffer (filled triangles). On day 28 the mice were challenged with 100 TCID50 of A/PR8 virus and weight was measured for 16 days. Mice were euthanized when weight loss exceeded 20%.*

Detailed comparison of the immune response in vaccinated mice revealed that WIV induces higher serum HI titers than SV and SU vaccine especially after a first immunization (refs. [26,27] and Chapters 2, and 3). These data are in line with the superior immunogenicity of WIV in unprimed individuals mentioned above. Interestingly, the different vaccine formulations also induce different types of responses. WIV vaccine elicits an overt Th1 type immune response characterized by high IgG2a/c titers and high numbers of IFNγ-producing T cells. In contrast, subunit and split vaccines induce primarily a Th2 response with large amounts of IgG1 and high numbers of IL4-producing T cells (refs. [26,27], and Chapters 2, and 3).

The relative contribution of IgG subtypes to protection from influenza challenge has been investigated in detail by Huber *et al* [28]. Mice were vaccinated with an HA DNA vaccine, with a viral replicon system encoding HA or with a combination of both to induce IgG1-dominated, IgG2a-dominated or mixed IgG1/IgG2a responses, respectively. IgG2a alone protected mice as efficiently from lethal

challenge as a mixture of IgG1 and IgG2a and could contain both mild and severe virus challenge. In contrast, IgG1 alone only protected from mild virus challenge but provided insufficient protection upon a high-dose challenge. Thus, antibody subclasses differ in their capacity to neutralize virus. As possible reasons for the superiority of IgG2a the affinity of this subtype for complement factors and Fc receptors has been proposed as both mechanisms contribute to efficient clearance of virus by phagocytosis and antibody-mediated cellular cytotoxicity [28]. In contrast, IgG1 does not lead to complement activation and has only weak affinity for Fc receptors.

Thus, current unadjuvanted vaccine formulations differ substantially in the magnitude but also in the phenotype of the immune reaction they evoke and this may have profound effects on their protective capacity at least in animal models. Data on the phenotype of the immune response to influenza vaccination and the relevance of the immune phenotype for protection in humans are urgently needed. Moreover, for a rational design of improved vaccines we need to understand which vaccine properties determine the differential immune reactions.

Mechanisms involved in vaccine-induced immunity

In order to reveal which mechanisms are responsible for the superior immunity of WIV, we and others have evaluated the effects of influenza vaccines on dendritic cells (DC) *in vitro*. Similar to live virus, WIV induced the production of IL12 and a variety of pro-inflammatory cytokines in conventional DC (cDC) generated from bone marrow by culture in the presence of GM-CSF (Chapter 2, and ref. [29]). In contrast, SV and SU vaccine had little effect. Plasmacytoid DC (pDC) were either generated from bone marrow by culture in the presence of Flt3-ligand or were directly purified from spleen. When incubated with live virus or WIV, these cells responded with production of type I interferons (IFN-α and IFN-β), while neither SV nor SU vaccine had any effect (Chapters 2, and 3, and ref. [30]).

Type I interferons are antiviral cytokines produced immediately upon viral infection. They have been shown to augment antibody responses *in vivo* and to steer the phenotype of the response to a Th1 type by direct interaction with B-cells, (but also T-cells) [31-34]. The role of type I interferons in the immune response to WIV was recently studied by Koyama and co-workers using IFNα/β receptor knock-out mice [30]. In contrast to wild type mice, IFNα/β receptor knock-out mice immunized with WIV showed little induction of antibodies and no induction

of influenza-specific IFNγ-producing Th cells and died quickly upon lethal virus challenge. Since pDC had been identified as an important source of type I IFN *in vitro*, the role of this cell type for the immune responses *in vivo* was investigated. Depletion of pDC by antibody treatment prior to intranasal immunization with WIV largely abolished IgG induction and significantly reduced induction of Th1 cells. Interestingly, pDCs were essential only during primary immunization while depletion prior to a booster immunization had no effect [30]. These results show that type I IFNs have an important role in the strong antibody and T cell response to immunization with WIV and that pDC as the major producers of type I IFNs are important, at least in the context of intranasal immunization. The role of pDC in the response to parenterally administered vaccines remains to be elucidated.

In the *in vitro* experiments described above only WIV but not SV or SU vaccine induced IFNα production by pDC (Chapters 2, and 3). WIV differs from the other vaccine formulations by retaining the structure of the virus envelope and by containing the viral RNA (which is degraded in SV and removed in SU vaccines). Although an intact envelope structure may enhance antigen uptake, it does not by itself trigger production of IFNα since pDC incubated with reconstituted viral envelopes (virosomes) do not secrete this cytokine (Chapter 2). Single-stranded RNA (ssRNA) is known as the ligand of Toll-like receptor (TLR) 7 which is expressed in pDC [35]. Indeed, bone-marrow derived Flt3-ligand pDC from TLR7 knock-out mice do not produce IFNα when incubated with WIV (Chapter 3, and ref. [30]). On the other hand, transfection of pDC with viral RNA purified from WIV leads to similar IFNα production as incubation with WIV itself (Chapter 3). Thus, binding of the viral ssRNA in WIV to TLR7 is the trigger for activation and IFNα production in pDC. TLR7 is also essential for immune responses to WIV *in vivo*. In TLR7 k.o. mice antibody induction to intramuscularly or intranasally administered WIV is significantly reduced or completely abolished, respectively, and Th1 induction is largely lost (Chapter 3, and ref. [30]).

Not only pDC but also B cells express TLR7 [36]. In fact, B cell-intrinsic TLR signaling was found recently to be essential for class switching to IgG2a/c in immunization and infection [37]. Moreover, B cells also possess IFNα/β receptors. Accordingly, B cells can react to viruses and vaccines carrying TLR7 ligands in a direct way by intrinsic TLR7 signalling as well as in an indirect way imposed by pDC-derived IFN-α [38]. Additionally, pDC-derived IFN-α can up-regulate the expression of TLR7 in B-cells, thereby increasing TLR7 sensitivity [39]. A third

signalling route can be provided by interactions between B cells and T cells via CD40-CD40L binding.

Thus, B cells receive a variety of signals either via direct contact with the antigen, via cell-cell contact with T cells and via cytokines derived from pDC and cDC. Heer and co-workers suggested a model illustrating how the different signaling mechanisms might be integrated and affect antibody production by B cells [38]. In this model, triggering of either TLR7 or CD40 on B cells alone induces cell proliferation and production of IgG1, whereas stimulation of both receptors together induces production of IgG1 and IgG2a/c antibodies. Additional signaling through the IFNα/β receptor further polarizes the subtype ratio in favor of IgG2a. Fig. 3 illustrates how WIV and SU vaccine would activate B cells in mice according to this model. WIV can activate B cells directly via cross-linking of B cell receptors and engagement of B cell-intrinsic TLR7. Via triggering of TLR7 in pDC it induces secretion of IFNα which in turn can further activate B cells. WIV also strongly stimulates cDC which produce cytokines like IL-12 and activate Th cells. By direct cell-cell contact between B and T cells involving CD40-CD40L binding and by cytokines derived from cDC and Th cells B cells are further activated. Integration of all these signals leads to B cell proliferation, class switching to IgG2a/c and strong production of antibodies. In contrast, SU vaccine does not activate B cells directly and does not involve pDC. Instead SU-activated cDC stimulate Th2-skewed Th responses via direct contact and via cytokines. Cell-cell contact between B cells and T cells and cytokines derived from cDC and Th cells induce B cell proliferation and production of antibodies of the IgG1 subclass. To what extent this model applies to the working mechanisms of WIV and SU vaccines in humans remains to be elucidated.

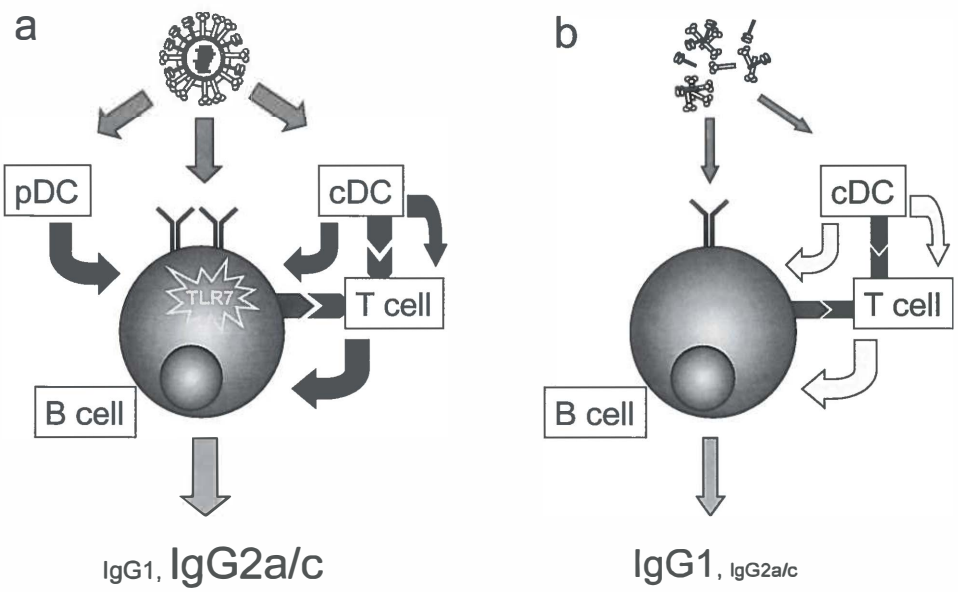

Figure 3. Model of B cell activation by WIV (a) or SU (b) vaccine. See text for explanation.

Adjuvants for influenza vaccines

The results obtained with WIV carrying TLR ligands versus SV or SU vaccines which are devoid of TLR ligands indicate that synthetic TLR ligands might be promising adjuvants for influenza vaccines. Indeed, the Koyama study shows that the lack of IFNα induction and immunogenicity of intranasally administered SV vaccine can be completely restored by addition of a CpG-based adjuvant [30]. Other TLR ligands investigated in the context of influenza vaccines include MPLA, flagellins, Poly I:C, and peptide-modified CpG (IC31) [30, 40-43]. Of these adjuvants, IC31 consisting of CpG oligomers bound to an immunostimulatory peptide has already successfully passed Phase I clinical testing [44]. Yet, no influenza vaccine adjuvanted with TLR ligands has obtained registration so far.

Adjuvants currently licensed for use with influenza vaccines comprise aluminium salts and the oil-in-water emulsions MF59 and AS03. Neither of these falls in the category of TLR ligands, rather these adjuvants work via other, so far only partly elucidated mechanisms.

Aluminium salts (phosphate or hydroxide) were introduced as adjuvants for human vaccines in 1932. Being components of diphtheria, tetanus, pertussis, and some *Haemophilus influenzae*, hepatitis A and hepatitis B vaccines, aluminium

salts are currently the principle adjuvants in clinical use [45]. Originally, aluminium adjuvants were thought to stimulate immune responses by providing a depot from which antigen is released slowly during an extended time period. Currently, activation of inflammasomes is considered as the primary mechanism of action [46]. Aluminium salts are known to stimulate predominantly Th2-type responses characterized by IL-4 and IL-5 production and the generation of antibodies of the IgG1 subtype in mice [46].

Clinical trials with aluminium-adjuvanted influenza vaccines in the past showed low to modest enhancing effects of the adjuvant on HI titers. More recently, aluminium adjuvants have been tested in the context of H5N1 prepandemic vaccines. These studies, which were performed with WIV as well as subvirion vaccines, rendered inconsistent results varying from modest enhancement to a decrease in antibody titers when unadjuvanted and adjuvanted vaccines were compared side-by-side [47,48]. Nevertheless, WIV influenza vaccines adjuvanted with aluminium salts were registered for use during the 2009 H1N1 pandemic in Hungary and in China [49]. No data are available on the effect of aluminium adjuvants on the protective efficacy of influenza vaccines in humans.

Influenza immunization studies in mice confirmed the known Th2-skewing properties of aluminium-based adjuvants. When combined with subunit vaccine, which by itself raised an IgG1-dominated antibody response, aluminium hydroxide increased HI and IgG1 titres. When combined with WIV, the IgG2a/c response usually evoked by this formulation was completely blocked. Instead, a strong IgG1 response was elicited which was correlated with enhanced HI titres. Yet, the efficacy of the vaccines to protect from severe virus challenge was not improved and was even decreased for aluminium-adjuvanted WIV (Chapter 5). The situation might be different for mild virus challenge as others reported improved protection after immunization with aluminium-adjuvanted WIV [50]. The differing results might thus be related to the fact that IgG1 alone can protect from mild virus challenge but that IgG2a is essential to contain the virus upon severe challenge as discussed above [28]. In any case, these results underline that the HI titer alone is insufficient as correlate of protection. Rather, qualitative parameters such as the phenotype of the elicited response also need to be evaluated and adjuvants used in combination with influenza should preferably support a mixed Th1/Th2 response or a Th1-dominated response.

Other adjuvants used clinically in combination with influenza vaccines are the water-in-oil emusions MF59 and AS03. A seasonal MF59-adjuvanted SV vaccine (Fluad®) is licensed in Europe for use in elderly and has been administered to more than 27 million subjects without causing significant side effects [51]. In clinical trials of H5N1 prepandemic vaccines, MF59 proved to enhance antibody titers, allow antigen dose reduction and, importantly, induce cross-reactive antibodies against drift strains [52-54]. The latter is explained by the fact that MF59 broadens the antibody response such that a larger number of HA epitopes is recognized [55]. An MF59-adjuvanted vaccine, Focetria®, was used during the 2009 H1N1 pandemic.

The working mechanism of MF59 is not entirely clear yet. Obviously, it involves enhanced recruitment of mononuclear cells to the injection site, promotion of differentiation of monocytes to dendritic cells with concomitant upregulation of costimulatory molecules, and enhanced antigen uptake [56]. In mice, MF59 induces a Th2 type immune response dominated by IgG1 and IL5-producing Th cells. Combination of MF59 with Th1-skewing adjuvants like CpG or synthetic TLR4 ligands might allow skewing of the response into the desired Th1 direction [57]. In ferrets, MF59 modestly improved the cross-protective potential of a WIV H5N1 vaccine. Yet, no further analysis of immune parameters was done [58]. In a clinical study, MF59 had little effect on the phenotype of the Th response to a subunit H5N1 vaccine, the response consisting mainly of $IL2^{+}IFN\gamma^{-}$ cells [59].

AS03, another oil-in-water adjuvant, was explored extensively in the context of H5N1 prepandemic vaccines (for recent review see [19]). AS03 has been used on a large scale as adjuvant in the 2009 H1N1 pandemic vaccine Pandemrix®, with a very high estimated efficacy of 98.6 and 83.3 in 14-59 year olds and > 60 year olds, respectively [60]. Similar to MF59, it enhances antibody titers, allows antigen dose sparing and elicits protection against homotypic and heterotypic influenza strains [61]. So far, the mechanism of action of AS03 and the immune phenotype elicited by AS03-adjuvanted influenza vaccines remain elusive.

Thus, currently used adjuvants were included in influenza vaccines with the aim of enhancing HI titers, the only correlate of protection generally accepted so far. Other parameters like the phenotype of the response induced and the effects of adjuvants in naïve versus primed individuals have only been investigated sporadically if at all. Taking these parameters into consideration might greatly enhance the benefits of future adjuvants.

Concluding remarks

So far, vaccine development, including the development of influenza vaccines, has taken place largely by a 'trial-and-error' approach. Undoubtedly, this approach has been successful in the sense that influenza vaccines with high efficacy are available at least for adolescents and young to middle-aged adults. Yet, implementation of recent insights in innate and adaptive immune response has the potential to significantly improve current vaccines in their protective efficacy with possibly lower amounts of antigen needed than used today. Such a more rational approach to vaccine design has found its way into vaccine development only recently.

Clues for vaccine improvement come from the detailed study of the immune responses to and the immune mechanisms involved by current vaccines. Animal experiments indicate that the value of HI titers as correlate of protection may be limited and, rather, knowledge of the immune phenotype is needed to judge the potential of influenza vaccines. In this context, immunological experience may be relevant. The majority of adolescents and adults will have developed a Th1 memory response to influenza virus caused by natural infection. In these subjects vaccination against seasonal influenza will probably boost this response without changing its phenotype. Things are different when young children need to be vaccinated or when vaccination against a new virus strain is necessary as in case of a pandemic. Results from mouse experiments imply that the phenotype imposed by priming might govern the response during later infection. Thus, a vaccine-induced Th2 response leads to an unnatural and suboptimal Th2 reaction during later influenza infection (A. Huckriede, unpublished observations). If this would hold true for the human situation, special care should be taken to induce the appropriate immune response in individuals who are immunologically naïve for the antigen in question. These observations indicate that it is highly important to learn more about the relevance of the immune phenotype in humans. Clinical evaluation of influenza vaccines should therefore include determination of immunoglobulin subclasses and/or Th cell profiles.

Regarding current knowledge, influenza vaccines should activate DC to secrete Th1-skewing cytokines like type I IFNs and IL-12. In this light, vaccines based on intact virus particles are interesting as they contain ssRNA as an intrinsic natural adjuvant with Th1-skweing properties. Live-attenuated vaccines as in use

in the US and the former Soviet Union fall in this category. Yet, in Europe these vaccines have so far not received approval due to safety concerns related to the possibility of reassortment between vaccine strain and circulating strains. WIV vaccines can be an alternative provided that reactogenicity, associated with early WIV vaccines, can be controlled. Post-marketing surveillance is now possible for the 2009 H1N1 WIV vaccine Celvapan® and should shed light on this issue.

Our knowledge about innate immunity and the interaction between innate and adapative immune responses has made enormous progress during the last 10 years. Moreover, much has been learned about influenza vaccines since the threat of an H5N1 pandemic boosted research activities. Implementation of this knowledge will be decisive for the development of safe and highly efficacious influenza vaccines needed to control seasonal influenza in an aging population and future influenza pandemics.

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References

1. Stöhr K, Kieny MP, Wood D. Influenza pandemic vaccines: how to ensure a low-cost, low-dose option. Nat Rev Microbiol 2006; 4:565-566.
2. Johnson NP, Mueller J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med 2002; 76:105-115.
3. Bautista E, Chotpitayasunondh T, Gao Z, Harper SA, Shaw M, Uyeki TM, Zaki SR, Hayden FG, Hui DS, Kettner JD, Kumar A, Lim M, Shindo N, Penn C, Nicholson KG. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza. N Engl J Med 2010; 362:1708-1719.
4. Taubenberger JK, Morens DM. The pathology of influenza virus infections. Annu Rev Pathol 2008; 3:499-522.
5. Rothbarth PH, Groen J, Bohnen AM, de Groot R, Osterhaus AD. Influenza virus serology--a comparative study. J Virol Methods 1999; 78:163-169.
6. Román E, Miller E, Harmsen A, Wiley J, Von Andrian UH, Huston G, Swain SL. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. J Exp Med 2002; 196:957-968.
7. Boon AC, Fringuelli E, Graus YM, Fouchier RA, Sintnicolaas K, Iorio AM, Rimmelzwaan GF, Osterhaus AD. Influenza A virus specific T cell immunity in humans during aging. Virology 2002; 299:100-108.
8. Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 2009; 459:931-939.
9. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. Nature 2005; 437:889-893.
10. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 2009; 360:2605-2615.
11. http://www.who.int/csr/disease/avian influenza/country/cases table 2010 06 08/en/index.html
12. Yang Y, Halloran ME, Sugimoto JD, Longini IM Jr. Detecting human-to-human transmission of avian influenza A (H5N1). Emerg Infect Dis 2007; 13:1348-1353.
13. Nichol KL, Treanor JJ. Vaccines for seasonal and pandemic influenza. J Infect Dis 2006; 194 Suppl 2:S111-118.
14. Wood JM, Williams MS History of inactivated influenza vaccines. In: Nicholson KG, Webster RG, Hay AJ, eds. Textbook of Influenza. Oxford: Blackwell Science. (1998) pp 317–323.
15. Ehrlich HJ, Müller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, Fisher D, Berezuk G, Fritsch S, Löw-Baselli A, Vartian N, Bobrovsky R, Pavlova BG, Pöllabauer EM, Kistner O, Barrett PN; Baxter H5N1 Pandemic Influenza Vaccine Clinical Study Team. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N Engl J Med 2008; 358:2573-2584.

16. Kistner O, Crowe BA, Wodal W, Kerschbaum A, Savidis-Dacho H, Sabarth N, Falkner FG, Mayerhofer I, Mundt W, Reiter M, Grillberger L, Tauer C, Graninger M, Sachslehner A, Schwendinger M, Brühl P, Kreil TR, Ehrlich HJ, Barrett PN. A whole virus pandemic influenza H1N1 vaccine is highly immunogenic and protective in active immunization and passive protection mouse models. PLoS One 2010; 5 :e9349.

17. Vajo Z, Tamas F, Sinka L, Jankovics I. Safety and immunogenicity of a 2009 pandemic influenza A H1N1 vaccine when administered alone or simultaneously with the seasonal influenza vaccine for the 2009-10 influenza season: a multicentre, randomised controlled trial. Lancet 2010; 375:49-55.

18. Waddington CS, Walker WT, Oeser C, Reiner A, John T, Wilkins S, Casey M, Eccleston PE, Allen RJ, Okike I, Ladhani S, Sheasby E, Hoschler K, Andrews N, Waight P, Collinson AC, Heath PT, Finn A, Faust SN, Snape MD, Miller E, Pollard AJ. Safety and immunogenicity of AS03B adjuvanted split virion versus non-adjuvanted whole virion H1N1 influenza vaccine in UK children aged 6 months-12 years: open label, randomised, parallel group, multicentre study. BMJ 2010; 340:c2649. doi: 10.1136/bmj.c2649.

19. Leroux-Roels G. Prepandemic H5N1 influenza vaccine adjuvanted with AS03: a review of the pre-clinical and clinical data. Expert Opin Biol Ther 2009; 9:1057-1071.

20. de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaus AD. Haemagglutination-inhibiting antibody to influenza virus. Dev Biol (Basel) 2003; 115:63-73.

21. Beyer WE, Palache AM, Osterhaus AD. Comparison of serology and reactogenicity between influenza subunit vaccines and whole virus or split vaccines: A review and meta-analysis of the literature. Clin Drug Investig 1998; 15:1–12.

22. Nicholson KG, Tyrrell DA, Harrison P, Potter CW, Jennings R, Clark A, Schild GC, Wood JM, Yetts R, Seagroatt V, Huggins A, Anderson SG. Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. J Biol Stand 1979; 7:123-136.

23. Stephenson I, Nicholson KG, Glück R, Mischler R, Newman RW, Palache AM, Verlander NQ, Warburton F, Wood JM, Zambon MC. Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. Lancet 2003; 362:1959-1966.

24. http://www.who.int/vaccine research/diseases/influenza/flu trials tables/en/

25. Hovden AO, Cox RJ, Madhun A, Haaheim LR. Two doses of parenterally administered split influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral shedding upon challenge in mice. Scand J Immunol 2005; 62: 342-352.

26. Hagenaars N, Mastrobattista E, Glansbeek H, Heldens J, van den Bosch H, Schijns V, Betbeder D, Vromans H, Jiskoot W. Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model. Vaccine 2008; 26: 6555-6563.

27. Hovden AO, Cox RJ, Haaheim LR. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol 2005; 62:36-44.

28. Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA, Makarova N, Perez DR, Macdonald GH, McCullers JA. Distinct contributions of vaccine-induced

immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol 2006; 13:981-990.

29. Saurwein-Teissl M, Zisterer K, Schmitt TL, Glück R, Cryz S, Grubeck-Loebenstein B. Whole virus influenza vaccine activates dendritic cells (DC) and stimulates cytokine production by peripheral blood mononuclear cells (PBMC) while subunit vaccines support T cell proliferation. Clin Exp Immunol 1998; 114:271-276.
30. Koyama S, Aoshi T, Tanimoto T, Kumagai Y, Kobiyama K, Tougan T, Sakurai K, Coban C, Horii T, Akira S, Ishii KJ. Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. Sci Transl Med 2010; 2:25ra24.
31. Proietti E, Bracci L, Puzelli S, Di Pucchio T, Sestili P, De Vincenzi E, Venditti M, Capone I, Seif I, De Maeyer E, Tough D, Donatelli I, Belardelli F. Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. J Immunol 2002; 169:375-383.
32. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity 2001; 14:461-470.
33. Coro ES, Chang WL, Baumgarth N. Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. J Immunol 2006; 176:4343-4351.
34. Chang WL, Coro ES, Rau FC, Xiao Y, Erle DJ, Baumgarth N. Influenza virus infection causes global respiratory tract B cell response modulation via innate immune signals. J Immunol 2007; 178:1457-1467.
35. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004; 303:1529-1531.
36. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, Endres S, Hartmann G. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002; 168:4531-4537.
37. Barr TA, Brown S, Mastroeni P, Gray D. B cell intrinsic MyD88 signals drive IFN-gamma production from T cells and control switching to IgG2c. J Immunol 2009; 183:1005-1012.
38. Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, Kopf M, Marsland BJ. TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. J Immunol 2007; 178:2182-2191.
39. Bekeredjian-Ding IB, Wagner M, Hornung V, Giese T, Schnurr M, Endres S, Hartmann G. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. J Immunol 2005; 174:4043-4050.
40. Miyaki C, Quintilio W, Miyaji EN, Botosso VF, Kubrusly FS, Santos FL, Iourtov D, Higashi HG, Raw I. Production of H5N1 (NIBRG-14) inactivated whole virus and split virion influenza vaccines and analysis of immunogenicity in mice using different adjuvant formulations. Vaccine 2010; 28:2505-2509.
41. Skountzou I, Martin Mdel P, Wang B, Ye L, Koutsonanos D, Weldon W, Jacob J, Compans RW. Salmonella flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. Vaccine 2010; 28:4103-4112.
42. Asahi-Ozaki Y, Itamura S, Ichinohe T, Strong P, Tamura S, Takahashi H, Sawa H, Moriyama M, Tashiro M, Sata T, Kurata T, Hasegawa H. Intranasal administration of adjuvant-

combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. Microbes Infect 2006; 8:2706-2714.

43. Riedl K, Riedl R, von Gabain A, Nagy E, Lingnau K. The novel adjuvant IC31 strongly improves influenza vaccine-specific cellular and humoral immune responses in young adult and aged mice. Vaccine 2008; 26:3461-3468.

44. http://www.intercell.com/main/forvaccperts/products/ic31R-seasonal-influenza-vaccine/.

45. Eickhoff TC, Myers M. Workshop summary. Aluminum in vaccines. Vaccine 2002; 20 Suppl 3:S1-4.

46. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol 2009; 9:287-293.

47. Keitel WA, Atmar RL. Vaccines for pandemic influenza: summary of recent clinical trials. Curr Top Microbiol Immunol 2009;333:431-51.

48. http://www.who.int/vaccine research/diseases/influenza/flu trials tables/en/index.html

49. http://ecdc.europa.eu/en/healthtopics/documents/0911 pandemic influenza (h1n1) qa on vaccines and vaccination experts.pdf

50. Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. Vaccine 2007; 25:3554-3560.

51. Schultze V, D'Agosto V, Wack A, Novicki D, Zorn J, Hennig R. Safety of MF59 adjuvant. Vaccine 2008; 26:3209-3222.

52. Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, Zambon MC. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 2001; 357:1937-1943.

53. Stephenson I, Bugarini R, Nicholson KG, Podda A, Wood JM, Zambon MC, Katz JM. Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza /Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. J Infect Dis 2005;191:1210-1215.

54. Banzhoff A, Gasparini R, Laghi-Pasini F, Staniscia T, Durando P, Montomoli E, Capecchi PL, di Giovanni P, Sticchi L, Gentile C, Hilbert A, Brauer V, Tilman S, Podda A. MF59-adjuvanted H5N1 vaccine induces immunologic memory and heterotypic antibody responses in non-elderly and elderly adults. PLoS One 2009; 4:e4384.

55. Khurana S, Chearwae W, Castellino F, Manischewitz J, King LR, Honorkiewicz A, Rock MT, Edwards KM, Del Giudice G, Rappuoli R, Golding H. Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. Sci Transl Med 2010; 2:15ra5.

56. Tritto E, Mosca F, De Gregorio E. Mechanism of action of licensed vaccine adjuvants. Vaccine 2009; 27:3331-3334.

57. Baudner BC, Ronconi V, Casini D, Tortoli M, Kazzaz J, Singh M, Hawkins LD, Wack A, O'Hagan DT. MF59 emulsion is an effective delivery system for a synthetic TLR4 agonist (E6020). Pharm Res 2009; 26:1477-1485.

58. Forrest HL, Khalenkov AM, Govorkova EA, Kim JK, Del Giudice G, Webster RG. Single- and multiple-clade influenza A H5N1 vaccines induce cross protection in ferrets. Vaccine. 2009; 27:4187-4195.

59. Galli G, Medini D, Borgogni E, Zedda L, Bardelli M, Malzone C, Nuti S, Tavarini S, Sammicheli C, Hilbert AK, Brauer V, Banzhoff A, Rappuoli R, Del Giudice G, Castellino F. Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels. Proc Natl Acad Sci U S A 2009; 106:3877-3882.

60. Wichmann O, Stocker P, Poggensee G, Altmann D, Walter D, Hellenbrand W, Krause G, Eckmanns T. Pandemic influenza A(H1N1) 2009 breakthrough infections and estimates of vaccine effectiveness in Germany 2009-2010. Euro Surveill. 2010; 15:pii: 19561.

61. Baras B, Stittelaar KJ, Simon JH, Thoolen RJ, Mossman SP, Pistoor FH, van Amerongen G, Wettendorff MA, Hanon E, Osterhaus AD. Cross-protection against lethal H5N1 challenge in ferrets with an adjuvanted pandemic influenza vaccine. PLoS One 2008; 3:e1401.

General Discussion Part II

Revival of whole inactivated virus vaccine for influenza pandemics

WIV vaccine has found renewed interest as (candidate) formulation in the context of an imminent H5N1 pandemic and the recent 2009 H1N1 pandemic [1-4]. Its response induction in naïve recipients is superior to other unadjuvanted formulations [5-8], which means that protective immunity may be established with a lower vaccine dose. Use of WIV can therefore be dose-sparing. Indeed, converting vaccine production to WIV is a strategy to increase vaccine availability, as was suggested by the WHO in response to the H5N1 pandemic threat [12]. In the past, WIV lost in popularity to less reactogenic split-virus and subunit vaccine. In a pandemic, however, reactogenicity is likely to be a minor problem [9]. Moreover, modern production technology may diminish WIV reactogenicity [10], and when compared to $ASO3_A$-adjuvanted split-virus vaccine used in the 2009 pandemic, WIV formulation was even better tolerated [11]. Recently, the number of companies having H5N1 and other subtype WIV vaccines under investigation has been increasing (up to 10) [13].

Yet, not everyone zeroed in on the revival of an archaic vaccine for pandemic use. At the height of H5N1 pandemic alertness in 2006, many companies would have gone with the vaccine type they were already producing [9]. Meanwhile, new powerful oil-in-water adjuvanted split-virus vaccines have been introduced, which seems to make the whole-versus-split virus issue less pressing than before. However, a recent head-to-head comparison between an H1N1 2009 pandemic $ASO3_A$-adjuvanted split-virus vaccine and an unadjuvanted whole-virus vaccine shows that the discussion is still alive [9,11,14]. The $ASO3_A$-adjuvanted split-virus vaccine showed better dose-sparing properties, but the outcome of the study was contested by others. Still, WIV may have special qualities that would favor it over split-virus and subunit vaccine as a basic formulation for a pandemic vaccine, especially if a proper adjuvant could be added.

Unique features of viral particles

Compared to split-virus and subunit vaccines, WIV has unique structural and functional features that may steer and augment the antibody response, as has been shown in mice (*Chapter 2*). Most importantly, WIV contains viral RNA that may act as an internal Th1-steering adjuvant, through interaction with TLR7 and induction of IFNα, as demonstrated in *Chapter 3* and discussed in detail in *Chapter 7*. Furthermore, the viral particle structure may serve to target the viral RNA to TLR7

in the endosomes, by facilitating receptor mediated uptake and protecting the RNA against degrading enzymes [15]. In absence of a particle structure, 'naked' influenza RNA has been shown to be a very inefficient stimulator of TLR7 *in vitro* [16]. Therefore, even if there would remain some viral RNA in split-virus vaccine after destruction of the particles (which is unlikely see *Chapter 3*), it may not be able to activate TLR7 efficiently.

Considering the above, the HA in WIV not only serves as the primary antigen like in split-virus and subunit vaccine, but may also function in the immune response by its binding capacity to the host immune cell, which facilitates receptor-mediated uptake. In addition, the HA in the viral particle facilitates viral membrane fusion, a function which remains intact in β-propiolactone-inactivated virus (*Chapter 3*) but might be abolished by (harsh) treatment with formalin (*Chapter 4* and reference 52). Theoretically, fusion activity of WIV could play a role in the antibody response following two lines of thought.

First, it was proposed by others that influenza viral membrane fusion could be important for efficient TLR7 signaling, through mechanisms unknown [17]. If TLR7 activation depends on membrane fusion, membrane fusion activity of WIV could be an important factor that co-determines the antibody response. Yet, we found that fusion activity of WIV had no effect on the serum quantities of influenza-specific IgG and Th1 type antibodies, as determined by ELISA. Loss of fusion activity led to moderately raised levels of Th2-type antibodies [*Chapter 4*]. Serum HI titers, assumed to be indicative of virus-neutralizing antibodies, seemed to be lower in mice immunized with fusion-inactive WIV. However, a change in the antigenic determinants resulting from formaldehyde treatment, as a method to inactivate fusion activity, may also play a role in this.

Second, membrane fusion may lead to efficient deposition of viral ssRNA in the cytoplasm of the recipient's immune cells, where it could stimulate RIG I-like receptors (RLR) of the innate immune system. RIG-I senses replicating influenza virus by its double stranded RNA intermediates, leading to production of IFNα [18]. However, RIG-I may also sense viral genomic ssRNA in absence of replication, through the formation of loops in the RNA molecules creating a double stranded 'panhandle' structure [19]. Stimulation of RIG-I, with concomitant IFNα production, by fusion active (but replication inactive) WIV could assist in Th1 steering of the immune response. In knock-out mice lacking both TLR7 and RIG-I signaling-function no Th1-type antibodies were found upon immunization with WIV [20], whereas low levels of Th1-type antibodies were

found in WIV-immunized TLR7 knock-out mice [20, and *Chapter 3*]. Yet, the role of RLR as compared to TLR7 in protective immunity seems to be small. After immunization with WIV, mice which lack RIG-I signalling are equally well protected as wild-type mice against a lethal viral challenge, whereas mice which lack TLR7 are not protected [21]. In conclusion, any contribution of fusion activity of WIV to the Th1-type antibody response, whether or not by facilitating RLR activation or by supposedly affecting TLR7 signalling, seems to be small [*Chapter 3 and 4*].

The process of viral uncoating, initiated by the low pH in the endosomes, has been linked to efficient TLR7 recognition of influenza virus [17,22]. Whether this process may play a role in the immune response to WIV is something which still needs to be studied. Low pH treatment of WIV prior to incubation with plasmacytoid dendritic cells (pDCs) leads to a strong reduction in IFNα production, while the binding capacity of WIV to MDCK cells does not seem to be affected (unpublished results). This might hint to a role for the proper timing of pH related effects on WIV, like viral uncoating, in the TLR7-mediated response. It may also suggest that potentially acidifying conditions should be avoided in the production process of WIV to assure maximal responses.

A role for WIV particles in priming

The advantage of having a machinery for effective TLR7 stimulation in case of WIV, seems highest when there is no pre-existing immunity, as would be in a pandemic caused by a virus which is unrelated to the circulating human virus strains, like avian H5N1. It has been shown that the activation of pDC's with subsequent IFN-alpha production plays a critical role in the primary response to an i.n. influenza WIV vaccine, while it does not seem to be involved in secondary responses [20]. In the primary response to vaccination, antibody-secreting cells and memory B-cells are formed in a process which usually includes antigen recognition, presentation and T cell help, and which may be augmented by co-stimulatory signals as provided by adjuvants or microbial vaccine components [38]. The response to WIV differs from that to split-virus and subunit vaccine by a strong activation of cDC and pDC *in vitro*, leading to production of proinflammatory cytokines and IFNα, as well as a strong induction of Th1 cells *in vivo* [*Chapter 2 and 3*]. These features will not be distinctive in a secondary

response, which merely requires the interaction between antigen and high affinity memory B-cells, the presence of which is accumulating after priming [38].

WIV and cross-protection

Cross-reactive anti-HA antibodies

Protective efficacy against drift variants of the vaccine virus is highly desirable for pandemic and epidemic vaccines, and a strong argument to maintain stockpiles of pre-pandemic H5N1 vaccines. Cross-reactive anti-HA antibodies recognizing drift variants of the H5N1 strain used for vaccination have been encountered with different pre-pandemic H5N1 vaccines in animals and humans [30-36]. In humans an H5N1 (clade 1) WIV vaccine induced substantial virus-neutralizing antibodies against clade 2 and 3 viruses (up to 45% and 78% seroconversion respectively). In contrast, H5N1 (clade 1) split-virus vaccine in two other studies induced neutralizing sero-conversion rates to a clade 2 virus that did not exceed 9% [30-32]. Cross-reactive neutralizing responses to heterologous virus induced by split-vaccine can be increased by the addition of oil-in-water adjuvants [31,32].

It is however unknown if the cross-neutralizing titers found in these studies would protect humans from a heterosubtypic H5N1 infection. In a mouse model WIV vaccine prepared from different H5N1 clades (1, 2.1, 2.2, 2.3.4) induced full or partial cross-protection against lethal infection with heterologous H5N1 viruses of different clades [36]. This was in part explained by the induction of cross-reactive antibodies, and it has been suggested that there is a common protective epitope shared by many different H5N1 viruses [36]. In ferrets similar cross-clade protection by H5N1 WIV vaccines, with or without the oil-in-water adjuvant MF59, against lethal infection has been observed [69].

Pre-pandemic vaccines may become even more effective in pandemic mitigation if the vaccines could also provide protection against shift variants. It has been shown in mice that an H3N2 WIV formulation fully protects against a lethal infection with H5N1, after 3 doses given in the presence of an adjuvants [37]. In the demonstrated cross-subtype protection B-cells seem to play a more important role than cytotoxic T-cells, likely by production of antibodies that recognize a common cross-reactive epitope on the HA shared by H3 and H5 [37].

The mechanism behind the induction of cross-reactive antibodies remains unclear. Cross-reactive antibodies may be directed to (neutralizing) epitopes located in the highly conserved stem domains of the HA (HA2) of different

subtypes [71], or possibly against some epitopes on the head domain of the HA (HA1) that are conserved between drift variants [66]. It has been shown that MF59-adjuvantation increases the production of antibodies recognizing HA1 rather than HA2. Adjuvantation also increased the avidity of these antibodies, as well as the diversity of epitopes that are recognized [70]. The increase in binding to HA1 epitopes correlated with broadening of cross-clade neutralization. MF59-adjuvantation strongly increases CD4+ T helper cell induction, and it has been suggested that this may be responsible for the broadening of the antibody response [66]. Obviously, adjuvantation plays a critical role in eliciting cross-reactive antibodies when using split-virus vaccine. It would be interesting to see whether the internal (RNA) adjuvant in WIV and TLR7 triggering, are important for cross-reactive antibody induction, but this seems rather likely.

Antibodies against M1, M2, and NP

The protein content in WIV and split-virus vaccine differs from subunit vaccine in that WIV and split-virus contain all of the viral proteins (except for NS1), whereas subunit only contains HA and NA. M1, M2 and NP are relatively conserved and induction of antibodies against these proteins is of special interest because it may contribute to heterosubtypic immunity [24,27-29].

Antibodies against M1 and M2 are produced upon infection but their contribution to virus neutralization is small compared to that of anti-HA [23]. Yet, induced by vaccination, anti-M2 has been shown to protect against lethal viral challenge [24], whereas immune-serum to M1 did not confer protection [25]. The mechanism of protection provided by antibodies against M2 is largely unclear. Possibly, expression of M2 on the cell surface and antibody-mediated cell cytotoxicity plays a role [68]. An anti-M2 monoclonal antibody has been shown to diminish viral spread in a plaque assay, but does not prevent infection [67].

Anti-NP antibodies were shown to be induced by WIV vaccination of mice [26]. Immunization of mice with large amounts of NP resulted in an antibody dependent reduction in morbidity and virus titers after viral challenge [27,28]. Anti-NP antibodies may protect in several ways. Anti-NP monoclonal antibodies can stimulate dendritic cell function and CTL induction through formation of immune complexes, and anti-NP IgG can induce complement mediated cytolysis, due to expression of NP on the cell surface [Summarized in 27].

Induction of cross-protective CTL

Stimulating CTL responses by vaccination may be another target in a pandemic situation. Compared to robust anti-viral antibody responses induction of cytotoxic T lymphocytes (CTL) seems negligible for protective immunity against influenza infection [50]. It is however believed that cross-protective CTLs may contribute to a reduction in morbidity and mortality when protective antibodies are absent. Such will be the case in an outbreak with a virus new to humans, like H5N1 [50].
In naïve mice formalin- as well as BPL-inactivated WIV induce CTLs, which may protect against challenge with homologous virus [51-53]. However, despite of the presence of conserved proteins in WIV and the capacity to induce CTL, intramuscular vaccination of mice with a formalin-inactivated seasonal influenza H3N2 strain did not deliver cross-protection against avian H5N1 [53]. The administration route as well as the method of WIV inactivation may play important roles in cross-protective CTL mediated immunity [52,54]. A recent study shows that intranasally delivered H1N1 WIV, inactivated by γ-irradiation, protected mice against a lethal infection with H5N1 with only a single dose [55]. The authors argue that CTLs are likely the cause of this heterosubtypic immunity. This demonstrates that induction of heterosubtypic immunity may be feasible using a WIV formulation, where this could not be shown for a commercial unadjuvanted split-virus vaccine [54].

Dose-sparing property of WIV

The limited vaccine production capacity makes strategies that minimize the dose of antigen needed for protection of utmost importance [12]. The rate-limiting step in vaccine production is the amount of virus that can be produced. Therefore, comparative evaluation of dose-sparing quality should at least take into account *the number of protective doses that can be produced from a given virus stock within a certain time*. It is for instance unknown how much HA is lost in the consecutive production steps to yield WIV, split-virus and subunit vaccine. Based on older research reports, loss of HA antigen by detergent and/or ether-treatment of whole virus may be estimated to be 10-30% [39-42]. More recently it was estimated that production of an H5N1 split-virus vaccine would lead to an HA loss of 20-30% [10] Even if this wastage could be strongly decreased by better production efficacy, a loss of only 1 % would mean that of the few billion pandemic doses envisaged, millions will be lost.

Also, as we have shown in *Chapter 5* (and discussed in *Chapter 7*), the quality of the immune response, in terms of Th1- or Th2-type response induction may be important to consider when evaluating a dose-sparing strategy. Until now a WIV formulation adjuvanted with aluminum-phosphate-gel has been the only H5N1 vaccine capable of meeting the European and U.S. licensing criteria using a *single* dose (of 6 μg HA) [43]. This may be very encouraging. We have, however, shown that aluminum-hydroxide skews the response to H1N1 WIV from a Th1 to a less effective Th2 type in mice [*Chapter 5*]. Whether this also applies to aluminum-phosphate-gel in the human situation remains to be seen. In contrast to our findings, another study in mice revealed a mixed Th1/Th2 response to an aluminum-hydroxide adjuvanted H5N1 WIV and better protective quality. The authors suggested that the vaccine dose, virus strain or the virulence of the virus might play a role in this discrepancy [46].

Addition of ASO3 to an H5N1 split-virus vaccine reduces the minimal antigen dose needed to meet European and FDA licensing criteria with more than 20 fold to 3.75 μg (in two doses), as compared to unadjuvanted split-virus [31, 32, 44]. The adjuvanted vaccine induced seroconversion in 82% of the recipients, compared to 4% without adjuvants. ASO3 seems to enhance both Th1 and Th2 responses [45]. To compare, 7.5 μg of an H5N1 WIV (in two doses) yielded a seroconversion rate of 69%, which was in the absence of an added adjuvants [30]. It would be very interesting to see if ASO3 could add to WIV's dose-sparing property, while preserving the Th1 response phenotype. In Sweden and Finland, an ASO3-adjuvanted H1N1 split-virus vaccine used during the 2009 pandemic has been associated with an increased risk of narcolepsy-cataplexy in children [72]. The causative mechanism remains to be resolved, but the presence of the ASO3 adjuvant may play a role [73]. It may turn out that strategies that minimize the dose of ASO3, for instance by combining ASO3 with WIV, could be favorable to reduce the risk of this rare side-effect.

MF59, another oil-in-water adjuvant [see also *Chapter 7*] with dose-sparing potential [47], is a Th2-steering adjuvant in mice [48], while in humans it may induce a Th1-type reponse [49]. Added to H5N1 WIV, MF59 enhances and skews the response in mice to a mixed Th1/Th2 type, which is superior in protection to unadjuvanted WIV or Al(OH)3 adjuvanted WIV, at least in type I diabetic mice [46].

WIV and vaccine stability

When it comes to timely production and distribution of billions of vaccine doses associated with a pandemic, logistics may become very complicated. A strong dependence on cold-chain requirements could hamper transportation and storage, and lead to vaccine losses due to cold-chain failures. H5N1 WIV vaccine was shown to be very stable and could be kept outside the fridge for at least a year without considerable losses in immunogenicity [*Chapter 6*]. WIV appeared to be more stable than subunit vaccine which lost potency under similar conditions after 3-5 months [56]. However, differences in vaccine virus and stability readout systems between these studies make a thorough comparison difficult.
The stability of WIV can be increased by freeze-drying and the use of sugar-glass technology, which allows storage at higher temperatures (40 degrees Celsius) for months [*Chapter 6*]. Considering the geographic distribution of human infections there is a fair chance that putative H5N1 outbreak containment actions may have to be performed in countries close to the equator, possibly in a rural area with poor infrastructure [57-59]. Moreover, use of dry-powder formulations may benefit the stockpiling of pre-pandemic vaccines performed by several countries and the WHO (for containment action and resource-poor countries) [60]. The improved stability of these vaccines creates longer shelf lives and might speed up vaccine deployment with minimal vaccine losses due to cold-chain failures.

Concluding remarks

During the last four decades evidence is accumulating that WIV vaccine induces stronger, better, broader and faster immune responses in unprimed individuals than other unadjuvanted inactivated vaccines [*Chapter 2 and 3*, references 5-8,61,62]. Its characteristic Th1-type response seems to be associated with better protection than Th2 type responses induced by the other vaccines [*Chapter 5*]. Production of WIV requires fewer production steps as compared to split-virus and subunit vaccines which would translate in a faster and cheaper production process, with minimal loss of HA, and requiring minimal technology and know-how. Additionally, WIV proved to be very stable in storage [*Chapter 6*]. An academic view on a solution for a worldwide problem posed by an imminent influenza pandemic may therefore favor inactivated whole influenza virus as the

antigen constituent of pandemic vaccines. On the other hand, it has been shown that addition of oil-in-water adjuvants, like MF59 and ASO3, to subunit and split vaccine increases and broadens the immune response to WIV-like proportions and maybe beyond [11,13,14]. Yet, the immunogenic capacity of WIV in combination with an adjuvant, other than aluminum, is only recently being investigated. These studies, all performed in animals, include MF59-adjuvanted WIV, WIV and CpG encapsulated into nanoparticles, cationic lipid/DNA complex-adjuvanted WIV, and CoVaccine HT-adjuvanted WIV, where the presence of the adjuvant increased the immune response in each study [46,63,64,65]. Yet, the ultimate comparative laboratory and clinical trials using the same (new) adjuvants combined with different basic formulations of HA, including WIV, needs to be awaited. WIV vaccine in combination with a proper adjuvant could in potential provide the best pragmatic solution for pandemics in the near future.

References

1. Ehrlich HJ, Müller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, Fisher D, Berezuk G, Fritsch S, Löw-Baselli A, Vartian N, Bobrovsky R, Pavlova BG, Pöllabauer EM, Kistner O, Barrett PN; Baxter H5N1 Pandemic Influenza Vaccine Clinical Study Team. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N Engl J Med 2008; 358:2573-2584.
2. Kistner O, Crowe BA, Wodal W, Kerschbaum A, Savidis-Dacho H, Sabarth N, Falkner FG, Mayerhofer I, Mundt W, Reiter M, Grillberger L, Tauer C, Graninger M, Sachslehner A, Schwendinger M, Brühl P, Kreil TR, Ehrlich HJ, Barrett PN. A whole virus pandemic influenza H1N1 vaccine is highly immunogenic and protective in active immunization and passive protection mouse models. PLoS One 2010; 5 :e9349.
3. Vajo Z, Tamas F, Sinka L, Jankovics I. Safety and immunogenicity of a 2009 pandemic influenza A H1N1 vaccine when administered alone or simultaneously with the seasonal influenza vaccine for the 2009-10 influenza season: a multicentre, randomised controlled trial. Lancet 2010; 375:49-55.
4. Waddington CS, Walker WT, Oeser C, Reiner A, John T, Wilkins S, Casey M, Eccleston PE, Allen RJ, Okike I, Ladhani S, Sheasby E, Hoschler K, Andrews N, Waight P, Collinson AC, Heath PT, Finn A, Faust SN, Snape MD, Miller E, Pollard AJ. Safety and immunogenicity of AS03B adjuvanted split virion versus non-adjuvanted whole virion H1N1 influenza vaccine in UK children aged 6 months-12 years: open label, randomised, parallel group, multicentre study. BMJ 2010; 340:c2649. doi: 10.1136/bmj.c2649.
5. Beyer WE, Palache AM, Osterhaus AD. Comparison of serology and reactogenicity between influenza subunit vaccines and whole virus or split vaccines: A review and meta-analysis of the literature. Clin Drug Investig 1998; 15:1–12.
6. Nicholson KG, Tyrrell DA, Harrison P, Potter CW, Jennings R, Clark A, Schild GC, Wood JM, Yetts R, Seagroatt V, Huggins A, Anderson SG. Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. J Biol Stand 1979; 7:123-136.
7. Stephenson I, Nicholson KG, Glück R, Mischler R, Newman RW, Palache AM, Verlander NQ, Warburton F, Wood JM, Zambon MC. Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. Lancet 2003; 362:1959-1966.
8. Parkman PD, Hopps HE, Rastogi SC, Meyer HM Jr. Summary of clinical trials of influenza virus vaccines in adults. J Infect Dis. 1977;136 Suppl:S722-30.
9. Dennis C. Flu-vaccine makers toil to boost supply. Nature. 2006;440(7088):1099.
10. Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, Gao Q, Zhang Z, Liu Y, Wang Z, Yang M, Sun R, Li C, Lin S, Ji M, Liu Y, Wang X, Wood J, Feng Z, Wang Y, Yin W. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet. 2006;368(9540):991-7.
11. Nicholson KG, Abrams KR, Batham S, Clark TW, Hoschler K, Lim WS, Medina MJ, Nguyen-Van-Tam JS, Read RC, Warren FC, Zambon M. Immunogenicity and safety of a two-dose schedule of whole-virion and AS03A-adjuvanted 2009 influenza A (H1N1)

vaccines: a randomised, multicentre, age-stratified, head-to-head trial. Lancet Infect Dis. 2011;11(2):91-101.

12. (who http://www.who.int/vaccines-documents/DocsPDF06/863.pdf, 2006).
13. http://www.who.int/vaccine research/diseases/influenza/flu trials tables/en/
14. Barrett PN, Ehrlich HJ. Adjuvanted or whole-virion vaccine for 2009 influenza A (H1N1). Lancet Infect Dis. 2011;11(7):496-7; author reply 497-8.
15. Diebold SS. Recognition of viral single-stranded RNA by Toll-like receptors. Adv Drug Deliv Rev. 2008;60(7):813-23.
16. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004;303(5663): 1529-31.
17. Wang JP, Liu P, Latz E, Golenbock DT, Finberg RW, Libraty DH. Flavivirus activation of plasmacytoid dendritic cells delineates key elements of TLR7 signaling beyond endosomal recognition. J Immunol. 2006;177(10):7114-21.
18. Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 5'-Triphosphate RNA is the ligand for RIG-I. Science. 2006;314(5801):994-7.
19. Rehwinkel J, Tan CP, Goubau D, Schulz O, Pichlmair A, Bier K, Robb N, Vreede F, Barclay W, Fodor E, Reis e Sousa C. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. Cell. 2010;140(3):397-408.
20. Koyama S, Aoshi T, Tanimoto T, Kumagai Y, Kobiyama K, Tougan T, Sakurai K, Coban C, Horii T, Akira S, Ishii KJ. Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. Sci Transl Med. 2010;2(25):25ra24.
21. Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, Uematsu S, Kawai T, Akira S. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol. 2007;179(7):4711-20.
22. Wang JP, Bowen GN, Padden C, Cerny A, Finberg RW, Newburger PE, Kurt-Jones EA. Toll-like receptor-mediated activation of neutrophils by influenza A virus. Blood. 2008;112(5): 2028-34.
23. Zhang R, Rong X, Pan W, Peng T. Determination of serum neutralization antibodies against seasonal influenza A strain H3N2 and the emerging strains 2009 H1N1 and avian H5N1. Scand J Infect Dis. 2011;43(3):216-20.
24. Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med. 1999;5(10): 1157-63.
25. Sui Z, Chen Q, Fang F, Zheng M, Chen Z. Cross-protection against influenza virus infection by intranasal administration of M1-based vaccine with chitosan as an adjuvant. Vaccine. 2010;28(48):7690-8.
26. Brett SJ, Dunlop L, Liew FY, Tite JP. Influence of the antigen delivery system on immunoglobulin isotype selection and cytokine production in response to influenza A nucleoprotein. Immunology. 1993;80(2):306-12.

27. Carragher DM, Kaminski DA, Moquin A, Hartson L, Randall TD. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. J Immunol. 2008;181(6):4168-76.

28. Lamere MW, Moquin A, Lee FE, Misra RS, Blair PJ, Haynes L, Randall TD, Lund FE, Kaminski DA. Regulation of antinucleoprotein IgG by systemic vaccination and its effect on influenza virus clearance. J Virol. 2011;85(10):5027-35.

29. Rangel-Moreno J, Carragher DM, Misra RS, Kusser K, Hartson L, Moquin A, Lund FE, Randall TD. B cells promote resistance to heterosubtypic strains of influenza via multiple mechanisms. J Immunol. 2008;180(1):454-63.

30. Ehrlich HJ, Muller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N Engl J Med 2008;358(24): 2573-84.

31. Leroux-Roels I, Borkowski A, Vanwolleghem T, Drame M, Clement F, Hons E, et al. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet 2007;370(9587):580-9.

32. Levie K, Leroux-Roels I, Hoppenbrouwers K, Kervyn AD, Vandermeulen C, Forgus S, et al. An adjuvanted, low-dose, pandemic influenza A (H5N1) vaccine candidate is safe, immunogenic, and induces cross-reactive immune responses in healthy adults. J Infect Dis 2008;198(5):642-9.

33. Baras B, Stittelaar KJ, Simon JH, Thoolen RJ, Mossman SP, Pistoor FH, et al. Cross-protection against lethal H5N1 challenge in ferrets with an adjuvanted pandemic influenza vaccine. PLoS One 2008;3(1):e1401.

34. Fazekas G, Martosne-Mendi R, Jankovics I, Szilvasy I, Vajo Z. Cross-reactive immunity to clade 2 strains of influenza virus A subtype H5N1 induced in adults and elderly patients by Fluval, a prototype pandemic influenza virus vaccine derived by reverse genetics, formulated with a phosphate adjuvant, and directed to clade 1 strains. Clin Vaccine Immunol 2009;16(4):437-43.

35. Govorkova EA, Webby RJ, Humberd J, Seiler JP, Webster RG. Immunization with reverse-genetics-produced H5N1 influenza vaccine protects ferrets against homologous and heterologous challenge. J Infect Dis 2006;194(2):159-67.

36. Murakami S, Iwasa A, Iwatsuki-Horimoto K, Ito M, Kiso M, Kida H, et al. Cross-clade protective immunity of H5N1 influenza vaccines in a mouse model. Vaccine 2008;26(50): 6398-404.

37. Tumpey TM, Renshaw M, Clements JD, Katz JM. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J Virol. 2001;75(11):5141-50.

38. Lambert PH, Liu M, Siegrist CA. Can successful vaccines teach us how to induce efficient protective immune responses? Nat Med. 2005;11(4 Suppl):S54-62.

39. Johannsen R, Moser H, Hinz J, Friesen HJ, Gruschkau H. Quantification of haemagglutinin of influenza Tween-ether split vaccines by immunodiffusion. Vaccine. 1985;3(3 Suppl): 235-40.

40. Bachmayer H, Liehl E, Schmidt G. Preparation and properties of a novel influenza subunit vaccine. Postgrad Med J. 1976;52(608):360-7.

41. Laver WG, Webster RG. Preparation and immunogenicity of a purified influenza virus haemagglutinin and neuraminidase subunit vaccine. Postgrad Med J. 1976 Jun;52(608): 373-8.

42. Hoyle L, Barker SM. Fractionation of the influenza virus with the object of producing subunit vaccine. Postgrad Med J. 1973;49(569):193-4.

43. Vajo Z, Wood J, Kosa L, Szilvasy I, Paragh G, Pauliny Z, Bartha K, Visontay I, Kis A, Jankovics I. A single-dose influenza A (H5N1) vaccine safe and immunogenic in adult and elderly patients: an approach to pandemic vaccine development. J Virol. 2010;84(3):1237-42.

44. Leroux-Roels I, Bernhard R, Gérard P, Dramé M, Hanon E, Leroux-Roels G. Broad Clade 2 cross-reactive immunity induced by an adjuvanted clade 1 rH5N1 pandemic influenza vaccine. PLoS One. 2008;3(2):e1665.

45. Morel S, Didierlaurent A, Bourguignon P, Delhaye S, Baras B, Jacob V, Planty C, Elouahabi A, Harvengt P, Carlsen H, Kielland A, Chomez P, Garçon N, Van Mechelen M. Adjuvant System AS03 containing α-tocopherol modulates innate immune response and leads to improved adaptive immunity. Vaccine. 2011;29(13):2461-73.

46. Wu J, Wang F, Fang F, Zhang W, Chang H, Zheng L, Chen Z. Superior protection provided by a single dose of MF59-adjuvanted whole inactivated H5N1 influenza vaccine in type 1 diabetic mice. Arch Virol. 2011;156(3):387-95.

47. Keitel W, Groth N, Lattanzi M, Praus M, Hilbert AK, Borkowski A, Tsai TF. Dose ranging of adjuvant and antigen in a cell culture H5N1 influenza vaccine: safety and immunogenicity of a phase 1/2 clinical trial. Vaccine. 2010;28(3):840-8.

48. Valensi JP, Carlson JR, Van Nest GA. Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. J Immunol. 1994;153(9):4029-39.

49. Galli G, Medini D, Borgogni E, Zedda L, Bardelli M, Malzone C, Nuti S, Tavarini S, Sammicheli C, Hilbert AK, Brauer V, Banzhoff A, Rappuoli R, Del Giudice G, Castellino F. Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels. Proc Natl Acad Sci U S A. 2009;106(10):3877-82.

50. Rimmelzwaan GF, Fouchier RA, Osterhaus AD. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. Curr Opin Biotechnol. 2007;18(6):529-36.

51. Mbawuike IN, Wyde PR. Induction of CD8+ cytotoxic T cells by immunization with killed influenza virus and effect of cholera toxin B subunit. Vaccine. 1993;11(12):1205-13.

52. Budimir N, Meijerhof T, Wilschut J, Huckriede A, de Haan A. The role of membrane fusion activity of a whole inactivated influenza virus vaccine in (re)activation of influenza-specific cytotoxic T lymphocytes. Vaccine. 2010;28(52):8280-7.

53. Bodewes R, Kreijtz JH, Hillaire ML, Geelhoed-Mieras MM, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Vaccination with whole inactivated virus vaccine affects the induction of heterosubtypic immunity against influenza virus A/H5N1 and immunodominance of virus-specific CD8+ T-cell responses in mice. J Gen Virol. 2010;91(Pt 7):1743-53.

54. Furuya Y, Regner M, Lobigs M, Koskinen A, Müllbacher A, Alsharifi M. Effect of inactivation method on the cross-protective immunity induced by whole 'killed' influenza A viruses and commercial vaccine preparations. J Gen Virol. 2010;91(Pt 6):1450-60.

55. Alsharifi M, Furuya Y, Bowden TR, Lobigs M, Koskinen A, Regner M, Trinidad L, Boyle DB, Müllbacher A. Intranasal flu vaccine protective against seasonal and H5N1 avian influenza infections. PLoS One. 2009;4(4):e5336.

56. Coenen F, Tolboom JT, Frijlink HW. Stability of influenza subunit vaccine. Does a couple of days outside the refrigerator matter? Vaccine. 2006;24:525–31.

57. http://www.who.int/csr/disease/avian influenza/country/cases table 2011 08 09/en/index.html

58. Chen JM, Chen JW, Dai JJ, Sun YX. A survey of human cases of H5N1 avian influenza reported by the WHO before June 2006 for infection control. Am J Infect Control. 2007;35(7):467-9.

59. Vong S, Ly S, Mardy S, Holl D, Buchy P. Environmental contamination during influenza A virus (H5N1) outbreaks, Cambodia, 2006. Emerg Infect Dis. 2008;14(8):1303-5.

60. Jennings LC, Monto AS, Chan PK, Szucs TD, Nicholson KG. Stockpiling prepandemic influenza vaccines: a new cornerstone of pandemic preparedness plans. Lancet Infect Dis. 2008;8(10):650-8.

61. Hovden AO, Cox RJ, Haaheim LR. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol. 2005;62(1):36-44.

62. Hagenaars N, Mastrobattista E, Glansbeek H, Heldens J, van den Bosch H, Schijns V, Betbeder D, Vromans H, Jiskoot W. Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model. Vaccine. 2008 2;26(51):6555-63.

63. Hong DK, Chang S, Botham CM, Giffon TD, Fairman J, Lewis DB. Cationic lipid/DNA complex-adjuvanted influenza A virus vaccination induces robust cross-protective immunity. J Virol. 2010;84(24):12691-702.

64. Huang MH, Lin SC, Hsiao CH, Chao HJ, Yang HR, Liao CC, Chuang PW, Wu HP, Huang CY, Leng CH, Liu SJ, Chen HW, Chou AH, Hu AY, Chong P. Emulsified nanoparticles containing inactivated influenza virus and CpG oligodeoxynucleotides critically influences the host immune responses in mice. PLoS One. 2010;5(8):e12279.

65. Bodewes R, Kreijtz JH, van Amerongen G, Geelhoed-Mieras MM, Verburgh RJ, Heldens JG, Bedwell J, van den Brand JM, Kuiken T, van Baalen CA, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. A single immunization with CoVaccine HT-adjuvanted H5N1 influenza virus vaccine induces protective cellular and humoral immune responses in ferrets. J Virol. 2010;84(16):7943-52.

66. Dormitzer PR, Galli G, Castellino F, Golding H, Khurana S, Del Giudice G, Rappuoli R. Influenza vaccine immunology. Immunol Rev. 2011;239(1):167-77.

67. Zebedee SL, Lamb RA. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J Virol. 1988;62(8):2762-72.

68. Fiers W, De Filette M, Birkett A, Neirynck S, Min Jou W. A "universal" human influenza A vaccine. Virus Res. 2004;103(1-2):173-6.

69. Forrest HL, Khalenkov AM, Govorkova EA, Kim JK, Del Giudice G, Webster RG. Single- and multiple-clade influenza A H5N1 vaccines induce cross protection in ferrets. Vaccine. 2009;27(31):4187-95.

70. Khurana S, Chearwae W, Castellino F, Manischewitz J, King LR, Honorkiewicz A, Rock MT, Edwards KM, Del Giudice G, Rappuoli R, Golding H. Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. Sci Transl Med. 2010;2(15):15ra5.

71. Throsby M, van den Brink E, Jongeneelen M, Poon LL, Alard P, Cornelissen L, Bakker A, Cox F, van Deventer E, Guan Y, Cinatl J, ter Meulen J, Lasters I, Carsetti R, Peiris M, de Kruif J, Goudsmit J. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One. 2008;3(12):e3942.

72. Occurrence of narcolepsy with cataplexy among children and adolescents in relation to the H1N1 pandemic and Pandemrix vaccinations – Results of a case inventory study by the MPA in Sweden during 2009–2010. Available from: http://www.lakemedelsverket.se/upload/nyheter/2011/Fallinventeringsrapport pandermrix 110630.pdf

73. Dauvilliers Y, Montplaisir J, Cochen V, Desautels A, Einen M, Lin L, Kawashima M, Bayard S, Monaca C, Tiberge M, Filipini D, Tripathy A, Nguyen BH, Kotagal S, Mignot E. Post-H1N1 narcolepsy-cataplexy. Sleep. 2010;33(11):1428-30.

Summary

The continuous threat of a new influenza pandemic urges the development of optimally effective vaccines for better pandemic preparedness. Many challenges accompany vaccine development for pandemics. These relate to factors such as the uncertainty of the timing and causative virus subtype of a next pandemic, the speed of global spreading of the virus, and limitations in the vaccine production capacity. One important challenge is the accomplishment of a dose-reduction to diminish the anticipated vaccine shortage. This may be achieved by use of a vaccine formulation that induces stronger and better immune responses than those generally induced by seasonal flu vaccines. Developing a completely new and better formulation is a risky and time-consuming process, while the threat of an H5N1 pandemic required immediate pandemic preparedness. We therefore set out to identify -among the non-adjuvanted vaccine formulations available for clinical use- the best formulation in terms of the quantity and quality of the induced antibody response and to clarify its working mechanism. We tried to further increase vaccine immunogenicity by adding an adjuvant, and to improve vaccine stability by freeze-drying, for storage in anticipation of a pandemic.

In a first set of experiments, we compared classic influenza vaccine formulations, in use for vaccination against seasonal influenza, in a head-to-head immunization trial in mice. Inactivated whole virus vaccine (WIV) was superior in inducing virus-specific antibodies, as compared to split-virus (SV), subunit (SU) or virosomal (VS) vaccine. The antibody response to WIV correlated with better neutralization of the virus in vitro (Chapter 2 and 3). Also, WIV induced a Th1-type immune response, characterized by high levels of IgG2a/c subtype antibodies in mice, in contrast to SV, SU or VS vaccine. The latter vaccines all induced a Th2-type response, in which IgG1 antibodies dominate. The presence of IgG2a/c correlates with better protection against a viral challenge than the presence of IgG1 antibodies, according to literature and also to our own experience (see Chapter 5). Inbred mice used in our experiments are genetically almost identical. We showed that WIV consistently induced a Th1-type response in two different inbred mouse strains with genetic predisposition for an antibody-dominated immuno-phenotype (Balb/c) and a cell-dominated immuno-phenotype (C57Bl/6), respectively. This holds promise that WIV might induce a similar response type in an outbred population, where more genetic variation exists (Chapter 2). Taken together, in naïve mice WIV proved superior in terms of magnitude and phenotype of the induced immune response. Based on these results, and results from animal and human studies by others that report of a

similar superiority of WIV above other classic influenza vaccines in naïve individuals, WIV may be favoured as candidate pandemic vaccine formulation.

Intrigued by the better potential of this oldest vaccine formulation (1945) amongst the current influenza vaccines, we set out to unravel the immunogenic mechanisms of WIV, hoping that insights into its superior response induction might aid future vaccine improvement. First of all, we found that the Th1 immuno-phenotype induced by immunization with WIV was associated with the induction of cytokines important for the adaptive immune response, including type I interferon (IFN), by dendritic cells (DCs) in vitro (Chapter 2). Because type I IFN is strongly induced by viral ssRNA upon recognition by TLR7 receptors of the innate immune system, we hypothesized a link between TLR7 and the superiority of the WIV-induced immune response. To test this, we immunized TLR7 knock-out mice with WIV, SV, and SU. In TLR7 k.o. mice the levels of virus-specific antibodies induced by WIV were significantly reduced as compared to wild-type mice, and the response type was reversed to a Th2 type (Chapter 3). This result showed that TLR7 plays a crucial role in the superiority of the WIV response, likely due to the presence of viral ssRNA in the core of the intact viral particles in WIV. In contrast, SV and SU vaccines contain only low amounts of viral ssRNA. A long-standing enigma as to why splitting of virus and purification of proteins for vaccine production lead to a loss of immunogenicity seems to be resolved.

Aside from the structural aspects of the viral particles, also functional aspects seem to be important for the response induction by WIV. To reach the endosomes of the target cell, where TLR7 is located, viral particles likely follow the route of natural infection that includes the receptor-mediated uptake by the host cell. Another function of intact viral particles is viral membrane fusion, which was linked by others to TLR7 activation. We tested the role of fusion activity of WIV on vaccine immunogenicity (Chapter 4). Inactivation of the fusion capacity of WIV was accomplished using formaldehyde. In vitro, fusion-inactive WIV showed a reduced but not abrogated capacity to activate TLR7. In vivo, fusion-inactive WIV did not differ much from fusion-active WIV in its capacity to induce a Th1-type antibody response in mice, as measured by ELISA. However, in the hemagglutination-inhibition assay (HI), which is the generally used read-out system for vaccine efficacy, sera from mice that had received fusion-inactive WIV showed somewhat lower antibody titers against native virus than sera from mice immunized with fusion-active WIV. This discrepancy was, in part, explained by a possible effect of the formaldehyde treatment on epitopes on the hemagglutinin

protein. Therefore, although fusion activity of WIV particles does not seem to play a major role in its immunogenicity, care should be taken when using formaldehyde to produce inactivated vaccines.

We considered increasing the immunogenicity of WIV by addition of an adjuvant, to further enhance its dose-sparing qualities. We tested aluminium hydroxide (Alum), which at that time was one of the few adjuvants licensed for use in influenza vaccines (Chapter 5). Although the addition of Alum increased the levels of hemagglutination-inhibiting antibodies induced by WIV, it also induced a shift from a Th1- to a Th2-type antibody profile, which proved to be of inferior protective quality in a virus challenge model. Alum therefore does not seem to have an additive value when combined with WIV for use in a pandemic. Moreover, this study showed that the quality of the immune response may be important to consider when evaluating a vaccine, instead of relying on HI titers alone.

Vaccine stability is an important issue when it comes to stockpiling vaccines for imminent pandemics, and when cold-chain requirements could hamper rapid vaccine distribution. We tested whether and for how long the immunogenicity of WIV remained intact at elevated temperatures (Chapter 6). When stored for a period of one year at room temperature WIV hardly lost immunogenicity as tested in mice. However, at 40°C the immunogenicity of WIV quickly deteriorated. Yet, by freeze-drying WIV after addition of a sugar excipient (trehalose or inulin), the imunogenicity of WIV, including the Th1-response type, could be maintained for at least 3 months of storage at 40°C.

Taken together, these studies support the potential of WIV as a pandemic vaccine candidate formulation. In Chapter 7 we discuss how the insights gained in the context of the here described studies combined with insights provided by others may facilitate rational vaccine design. In Chapter 8 we discuss the further merits of WIV for its use in pandemics. Future research should reveal whether the immunogenicity of WIV can be enhanced, for instance by finding a proper adjuvant, or by exploiting the knowledge of its working mechanism, to improve pandemic vaccines and increase their worldwide availability.

Based on the results described in this thesis we can give the following recommendations for pandemic influenza vaccines: Of the current vaccine formulations WIV should be preferred as formulation in non-adjuvanted vaccines. Alum should not be used as an adjuvant in combination with WIV. Instead, new

adjuvants should be tested, preferably with different basic vaccine formulations including WIV, to decide which adjuvanted formulation is optimal for use in a pandemic. The quality of the immune response should be included when measuring vaccine efficacy. WIV may be preferred for stockpiling vaccines because of its good stability. To further increase vaccine stability, and allow storage at high temperatures, WIV may be freeze-dried with a sugar excipient. Interactions with the innate immune system, which critically determine the superiority of WIV, as well as structural and functional properties of viral particles and their components, may be further exploited for vaccine improvement.

Samenvatting in het Nederlands

Introductie

Het influenza virus is de verwekker van de griep en veroorzaakt in de wintermaanden epidemieën op het noordelijk halfrond. Sporadisch veroorzaakt het influenza virus een pandemie, waarbij het virus zich binnen korte tijd wereldwijd verspreidt met extra ziekte- en sterftegevallen tot gevolg. Tijdens de 'Spaanse griep' pandemie van 1918 zijn naar schatting tussen 50 en 100 miljoen mensen omgekomen. Het ontstaan van een pandemie hangt samen met de introductie van een nieuw subtype influenza virus vanuit het dierenrijk in de menselijke populatie. Er bestaat dan nog geen immuniteit tegen dit nieuwe virus, waardoor het zich snel kan verspreiden. Het is onvoorspelbaar wanneer zo'n introductie plaats gaat vinden, en welk subtype influenza virus dit zal betreffen, maar dat het in de toekomst weer een keer gaat gebeuren is zeer waarschijnlijk.

De uitbraken van het dodelijke H5N1 vogelgriep virus onder pluimvee in Zuidoost- Azie, waarbij ook mensen geïnfecteerd raakten, vormden een directe pandemische dreiging. De eerste uitbraak vond plaats in 1997. Sinds 2003 is het virus echter niet meer weggeweest en heeft zich in die tijd verspreid over meerdere continenten. De wereld gezondheidsorganisatie (WHO) hanteert nog steeds een verhoogde alarmfase, één stap onder het niveau waarbij overdraagbaarheid van mens naar mens gesignaleerd wordt. Tot nu overleed 60% van de mensen met een bewezen H5N1 infectie. Als er een air-borne H5N1 virus ontstaat dat van mens naar mens overdraagbaar is -dit kan door mutaties van het virus of door vermenging (reassortment) met een menselijk influenza virus- kan dit tot een levensgevaarlijke pandemie leiden.

Vaccinatie biedt de beste bescherming tegen infectie met het influenza virus. Echter, het ontwikkelen van een vaccin voor een eerstvolgende pandemie wordt bemoeilijkt door tal van zaken. Hieronder vallen de onzekerheid over de exacte identiteit (subtype) van het nieuwe pandemische virus, de onbekendheid van het tijdstip waarop de pandemie ontstaat, en de snelle wereldwijde verspreiding van het virus. Daarnaast is de totale vaccin productiecapaciteit gelimiteerd en zijn de productiefaciliteiten gesitueerd in met name geïndustrialiseerde landen. Tijdens een pandemie kan daarom niet iedereen ter wereld tijdig over vaccins beschikken. Het vergroten van de beschikbaarheid van vaccins is dan ook een van de grote uitdagingen voor vaccinonderzoekers. In principe zouden door een verkleining van de benodigde dosis per vaccin, meer vaccindoses in dezelfde tijd geproduceerd kunnen worden. Met dit idee is er in

het laatste decennium een sterke focus geweest op het verbeteren van de immunogeniciteit van het vaccin; een sterkere immuunrespons geeft betere bescherming bij een lagere vaccindosis. De oplossing kan worden gezocht in de formulering van het vaccin zelf en in de toevoeging van een adjuvans, een stof die het immuunsysteem extra stimuleert. Het vinden van de beste dosis-sparende vaccins is een race die nog steeds gelopen wordt.

Dit proefschrift

Doelstelling

Het doel van het onderzoek dat wordt beschreven in dit proefschrift was in eerste instantie het identificeren van de beste formulering voor een pandemisch vaccin in het scala van formuleringen die al in productie zijn voor de seizoensgriep. Het ontwikkelen van een compleet nieuwe formulering kost namelijk tijd, terwijl de dreiging van een H5N1 pandemie juist een onmiddellijke paraatheid vereist. Hiervoor hebben wij in een muismodel de immuunrespons tegen de verschillende vaccinformuleringen vergeleken, waarbij we gelet hebben op niet alleen hoogte (kwantiteit) van de antistof respons maar ook naar het type (kwaliteit) van de respons. Het volgende doel was het vinden van de verklaring voor de waargenomen verschillen in de immuunrespons tegen de verschillende formuleringen aan de hand van structurele en functionele aspecten van die formuleringen. Het laatste doel was de pandemische toepasbaarheid van de beste formulering te verbeteren door de immunogeniciteit verder te vergroten met behulp van een adjuvans, en door het vaccin te stabiliseren zodat het beter opgeslagen kan worden.

Verschillende formuleringen, verschillende antistofresponsen

Voor de productie van vaccins voor de seizoensgriep zijn verschillende formuleringen in gebruik. Wat deze formuleringen met elkaar gemeen hebben is dat ze de belangrijkste virale antigenen bevatten. Antigenen zijn eiwitten waartegen de beschermende antistoffen opgewekt worden. Voor het influenza virus zijn dit het haemagglutinine (HA) en het neuraminidase (NA) eiwit. Deze bevinden zich aan de buitenkant van het virus partikel, en zijn dus goed

'zichtbaar'voor het immuunsysteem. Om aan die eiwitten te komen wordt eerst, ongeacht welke van de gangbare formuleringen gebruikt gaat worden, een bulk aan virus geweekt. Dit virus wordt vervolgens chemisch geïnactiveerd. De oudste formulering dateert uit 1945 en betreft een vaccin dat hele geïnactiveerde virussen bevat (whole inactivated virus, WIV). Toevoeging van een detergens aan WIV vernietigt de structuur van het virus partikel en geeft een zogenaamd split-virus vaccin (SV). Verdere zuivering van het HA en NA geeft een subunit vaccin (SU). In een virosomen vaccin (VS) is eerst de virale kern met het genetisch materiaal van het virus, het virale RNA, verwijderd en vervolgens de structuur van het virus hersteld. Deze verder 'lege' virus partikels bevatten wel het HA en het NA.

Om te achterhalen wat de beste formulering is hebben wij groepen muizen geïmmuniseerd met de verschillende vaccinformuleringen (Hoofdstuk 2). WIV induceerde hogere antistof (IgG) titers dan SV, SU en VS in muizen, wat correleerde met een betere neutralisatie van het virus in vitro (Hoofdstuk 2 en 3). Hierbij werden hoge titers van het IgG2a/c subtype aangetroffen en relatief lage titers IgG1. Dit komt overeen met een Th1-type respons. Ook het verhoogde aantal gamma-interferon producerende cellen in de milten van de muizen duidt hierop. Dit is het type respons dat in het algemeen door virusinfecties geïnduceerd wordt. IgG2a/c antistoffen lijken geassocieerd te zijn met betere bescherming tegen infectie vergeleken met IgG1, zoals blijkt uit de literatuur en onze eigen ervaring (zie Hoofdstuk 5). SV, SU en VS induceerden Th2-type responsen waarin het IgG1 subtype antistof domineert. Dus behalve dat WIV vaccins minder bewerkelijk in de productie zijn geven ze, tenminste in muizen, een kwantitatief en kwalitatief betere respons en zouden ze daarom in principe lager gedoseerd kunnen worden, dan de andere vaccins. Dit is in lijn met bevindingen van andere onderzoeksgroepen, niet alleen in diermodellen maar ook in de mens. Op basis van deze gegevens zou WIV de beste optie zijn voor een pandemisch vaccin.

Sinds 1960-70 zijn de SV en SU vaccins meer in trek voor de seizoensgriep, vanwege een wat lager bijwerkingenprofiel ten opzichte van WIV. Echter, bij productie van SV en SU gaat een deel van de immunogeniciteit verloren, wat vooralsnog onbegrepen was. SV en SU vaccins induceren een lagere immuunrespons dan WIV in individuen die niet eerder in contact zijn geweest met het subtype virus waarvoor geïmmuniseerd wordt (en dus immunologisch naïef zijn, net zoals de muizen in bovengenoemde experimenten). Dit is niet het geval tijdens een seizoensgriep epidemie, maar wel tijdens een pandemie. In een rapport van de WHO wordt een switch naar de productie van WIV in de

vaccinindustrie als een reële mogelijkheid gezien om de wereldwijde beschikbaarheid van vaccins te vergroten.

ONTRAFELEN VAN HET WERKINGSMECHANISME VAN WIV

We begonnen met het ontrafelen van het werkingsmechanisme achter de superieure immunogeniciteit van WIV, met het idee dat de kennis daaromtrent bij zou kunnen dragen aan het verbeteren van vaccins. Allereerst vonden wij in vitro dat de inductie van een Th1-type respons door immunisatie van muizen met WIV samen ging met de productie van bepaalde cytokinen, stoffen, die belangrijk zijn voor de adaptieve antistofrespons. Met name interferon-alpha (IFNα), een cytokine met anti-virale werking, werd sterk geïnduceerd (Hoofdstuk 2). Het was eerder aangetoond dat viraal RNA de productie van IFNα stimuleert door Toll-like receptor 7 (TLR7) te activeren. TLR7 is een van de zogenaamde 'pattern recognition' receptoren van het aangeboren immuunsysteem, die verschillende grove structuren van infecterende micro-organismen herkennen, zoals bijvoorbeeld het RNA of DNA (TLR9). We stelden de hypothese dat de superieure immunogeniciteit van WIV afhangt van de activatie van TLR7. In de kernen van de geïnactiveerde virus partikels in WIV bevindt zich namelijk nog steeds het virale RNA genoom. Om de hypothese te toetsen hebben we WIV, SV en SU naast elkaar getest in muizen die deficiënt zijn gemaakt voor TLR7 en in wildtype muizen. We zagen dat de antistofrespons tegen WIV in TLR k.o. muizen ten opzichte van wildtype muizen niet alleen significant gereduceerd was, maar ook dat de respons-type veranderd was van een Th1-type in een Th2-type (Hoofdstuk 3). TLR7-activatie blijkt dus in grote mate verantwoordelijk voor de superieure immunogeniciteit van WIV ten opzichte van de andere formuleringen. Experimenten van anderen ondersteunen deze bevinding. Het blijkt dat de hoeveelheid RNA in SV en SU vaccin erg laag is ten opzichte van WIV. Hiermee lijkt het oude raadsel van het verlies aan immunogeniciteit door van WIV een SV of SU vaccin te maken opgelost. Tevens tonen onze resultaten aan dat het aangeboren immuunsysteem belangrijk is voor de activatie en sturing van de adaptieve immuunrespons tegen vaccins.

De rol van de partikelstructuur en fusogene eigenschappen van WIV

Naast de aanwezigheid van RNA in de viruspartikels lijken ook functionele aspecten van de partikels belangrijk voor de immuunrespons op WIV. Om bij TLR7 in de endosomen te komen moeten de viruspartikels eerst op natuurlijke wijze via receptoren aan de gastheercel binden en actief opgenomen worden. In dezelfde endosomen waar TLR7-activatie plaats vindt versmelt (fuseert) een deel van de viruspartikels met de gastheercel. Fusie-activiteit is een functie van het viruspartikel, en TLR7-activatie is door andere onderzoekers in verband gebracht met deze fusie-activiteit. Stappen in het productieproces van WIV die de fusie-activiteit zouden kunnen beïnvloeden, zoals het gebruik van formaldehyde om virus te inactiveren, zouden dus de immunogniciteit van WIV kunnen compromitteren. Of dit zo is hebben we getest door muizen te immuniseren met WIV nadat we het eerst fusie-inactief hadden gemaakt door middel van formaldehyde behandeling (Hoofdstuk 4). De fusie-activiteit lijkt evenwel geen invloed te hebben op de aantallen en de typen antistoffen die geproduceerd worden, gemeten met de ELISA test. Daarentegen vallen de aantallen antistoffen gemeten met de haemagglutinatie inhibitie (HI) test beduidend lager uit in geval van fusie-inactief WIV. Het laatste kon grotendeels verklaard worden door een effect van formaldehyde op de HI epitopen, en heeft waarschijnlijk niets met fusie-activiteit te maken. In vitro was de TLR7-activatie wel verminderd is (alleen bij de hoogste concentratie WIV) maar zeker niet nul. Dus fusie-activiteit lijkt geen belangrijke rol te spelen, maar voorzichtigheid lijkt geboden bij gebruik van formaldehyde om vaccines te inactiveren.

Kan WIV verbeterd worden met een adjuvans?

Het toevoegen van een adjuvans is een belangrijk manier om de immunogeniciteit van laag-immunogene vaccins te vergroten. Inmiddels zijn er krachtige olie-in-water adjuvantia voor influenza vaccins op de markt die dat ook daadwerkelijk kunnen doen. Wij hebben WIV in combinatie met aluminium-hydroxide (Alum) - een van de weinige adjuvantia met een gebruikslicentie voor influenza vaccins op dat moment- getest in een muismodel (Hoofdstuk 5). Echter, toevoeging van Alum ging gepaard met een shift van een Th1 naar een Th2-type antistofrespons.

Ondanks dat de muizen hogere HI antistof titers genereerden tegen WIV in combinatie met Alum, dan tegen WIV alleen, bleken ze minder goed beschermd tegen een challenge met actief virus in de neus. Dit onderzoek toonde aan dat gebruik van Alum in combinatie met WIV niet zinvol is, zoals ook is gebleken uit klinische studies. Tijdens de pandemie van 2009 ('Mexicaanse griep') zijn geadjuvanteerde SV of SU vaccins en WIV naast elkaar gebruikt. Volgens een recente studie zou de dosis-sparende capaciteit van een olie-in-water geadjuvanteerd SV vaccin beter zijn dan die van WIV vaccin. De vraag is hoe deze verhouding wordt als aan WIV ook een goed werkend adjuvans zou worden toegevoegd (zie Hoofdstuk 8).

VERBETEREN VAN DE STABILITEIT VAN WIV

Het opslaan van vaccins, in anticipatie op een mogelijke pandemie, kreeg veel aandacht toen bleek dat zogenaamde pre-pandemische H5N1 vaccins toch aanzienlijke bescherming geven tegen drift-varianten van het H5N1 virus. Dit zijn H5N1 virus stammen die ontstaan zijn als gevolg van kleine mutaties in de antigenen. Deze treden vaker op naarmate er meer tijd verstrijkt tussen de productie van het pre-pandemisch vaccin en het daadwerkelijk optreden van de pandemie. Voor menig vaccin geldt dat de houdbaarheidsdatum van een jaar is gesteld voor opslag bij 4° C. Wij hebben de stabiliteit van WIV onderzocht na opslag bij hogere temperaturen (Hoofdstuk 6). WIV liet na één jaar opslag bij kamertemperatuur nauwelijks verval in de immunogeniciteit zien. De stabiliteit van WIV na opslag bij 40° C bleek beduidend minder; na 3 maanden trad een aanzienlijke verval in de antilichaam respons op. Door WIV te vriesdrogen in een matrix van suikers, ook wel suikerglas techniek genaamd, bleef de immunogeniciteit van het vaccin bij deze extreme temperatuur wel behouden, inclusief de inductie van een Th1-type respons, voor een opslagduur van ten minste 3 maanden bij 40° C. De stabiliteit van WIV kan dus effectief vergroot worden door vriesdrogen in suikerglas. Gevriesdroogd WIV is dus minder afhankelijk van de koude-keten (cold-chain) en beter inzetbaarheid voor opslag en snelle verspreiding van vaccins ten tijde van een pandemie, met name in landen met een warmer klimaat.

AANBEVELINGEN

Op basis van de in dit proefschrift beschreven resultaten kunnen wij de volgende aanbevelingen geven ten aanzien van pandemische influenza vaccins: Van de huidige vaccinformuleringen zou WIV geprefereerd moeten worden als formulering in niet-geadjuvanteerde vaccins. Aluminium hydroxide zou niet gebruikt moeten worden als adjuvans in combinatie met WIV.. Nieuwe adjuvantia zouden getest moeten worden met verschillende van de huidige formuleringen, inclusief WIV, om uit te maken welke geadjuvanteerde formulering het best in een pandemie gebruikt kan gaan worden. Bij het meten van de effectiviteit van een vaccin zou ook gekeken moeten worden naar de kwaliteit van de immuunrespons. Een WIV formulering zou gekozen moeten worden voor opslag van vaccins vanwege een goede stabiliteit. Om de stabiliteit verder te vergroten en opslag bij hoge temperaturen mogelijk te maken kan WIV gevriesdroogd worden met een suiker bestanddeel. Interacties met het aangeboren immuunsysteem, waarvan we aangetoond hebben dat die van groot belang zijn voor de superieure immunogeniciteit van WIV, als wel de structurele en functionele eigenschappen van de virus partikel met zijn componenten, zouden verder benut kunnen worden om vaccins te verbeteren.

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Dankwoord

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